Cancer Treatment Reports

A Publication of the National Cancer Institute

Volume 71
Number 4
April 1987

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service National Institutes of Health

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**From* Methodologic Guidelines for Reports of Clinical Trials (Cancer Treat Rep 69:1-3, 1985).

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Action of 7-Ethylcamptothecin on Tumor Cells and Its Disposition in Mice[1]

Hiroshi Nagata,[2,3,*] Norimasa Kaneda,[2] Tomio Furuta,[2] Seigo Sawada,[2] Teruo Yokokura,[2] Tadashi Miyasaka,[4] Masako Fukada,[3] and Kunihiro Notake[3,5]

Some biological effects of camptothecin (CPT) and its new derivative 7-ethylcamptothecin (ECPT) were studied. The drugs were effective against murine leukemia; ECPT was more effective than CPT. Ip administration of ECPT or CPT gave maximum treated/control values of 325% and 232%, respectively. The drugs also inhibited the growth of KB cells in vitro, 50% effective doses of 3.5 ng/ml of ECPT and 8.6 ng/ml of CPT, indicating the stronger activity of ECPT. Pharmacokinetic studies of the drugs in mice showed that ECPT had a longer biological half-life in the terminal phase and a larger amount remained in the plasma compared with CPT. After iv administration of ECPT, the drug accumulated in the intestine, suggesting that the main route of excretion of the drug is through the biliary tract. The study on cell cycle progression by flow cytometry suggested that the main effect of both drugs on L1210 cells was the blocking of G_2-M phase. These results suggest that the main reasons for the superior antitumor activity of ECPT compared with CPT are as follows: (*a*) ECPT had a stronger growth-inhibiting activity against tumor cells, and (*b*) ECPT remained in the intestinal tract for a longer time and in higher amounts when administered in vivo. [Cancer Treat Rep 71:341-348, 1987]

Camptothecin (CPT), an alkaloid with a novel ring structure isolated from the Chinese tree *Camptotheca acuminata* (1), has shown antitumor effects on experimental neoplasms in vivo (1,2) and in vitro (3,4) and also on various human tumor stem cells in vitro (5). It inhibits the synthesis of DNA and RNA (6-8) and causes single-strand breakage (9,10) by alkali-labile linkage to DNA (11). Although CPT produces favorable results in animal studies, clinical studies have not been fruitful because of its low therapeutic efficacy and severe toxicity for the intestinal mucosa, bladder, and hematological system (12-16). Therefore, there have been less toxic and more effective derivatives produced such as 10-hydroxycamptothecin, whose biological activities have been studied (17,18). We also synthesized and screened various derivatives and found that 7-ethylcamptothecin (ECPT) has a stronger antitumor effect and is less toxic than CPT (19). In this paper, we present some biological effects of ECPT in vivo and in vitro.

MATERIALS AND METHODS

Animals and Drugs

BDF_1 female mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan) and CDF_1 female mice were purchased from Charles River Japan Inc (Atsugi, Japan). The animals were allowed food and water ad libitum except for the distribution study when animals were fasted overnight. All experiments were carried out on animals 7-10 weeks of age.

Mitomycin (MMC) and 5-fluorouracil (5-FU) were purchased from Kyowa Hakko Kogyo (Tokyo, Japan). ECPT (fig 1) was obtained as described previously (20).

Antitumor Activity

BDF_1 female mice (7 weeks old) were inoculated with L1210 cells (5×10^5/mouse) ip on Day 0 and given

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[5] We thank the Japan Spectroscopic Co, Ltd, for setting up the measurement of DNA distribution with the FCS-1 cell sorter and Dr. M. Mutai for his encouragement and support.
* *Reprint requests to:* Hiroshi Nagata, Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi-shi, Tokyo 186, Japan.

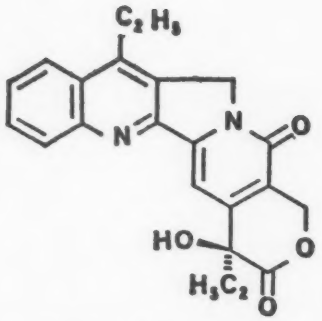

FIGURE 1.—Structure of ECPT.

ECPT or CPT iv or ip on Days 1, 5, and 9, for a total dose of 5–120 mg/kg (ten mice per group). Relative survival time of the animals was represented as treated (T)/control (C) (%) and was calculated as follows: T/C (%) = [(mean survival days of drug-treated mice)/(mean survival time of control mice)] × 100. The statistical significance of the data was determined by Student's t test.

Growth Inhibition Assay In Vitro

KB cells were grown at 37°C in Eagle's minimal essential medium with 10% calf serum (GIBCO Laboratories, Grand Island, NY). Cells (6×10^4) were placed in a plastic Petri dish (6 cm in diameter, Falcon) on Day −1 and incubated overnight. On Day 0, the medium was discarded and replaced with drug-containing fresh medium and the cells were incubated for an additional 3 days. ECPT and CPT were used as the sodium salt dissolved in water or were dissolved in 100% dimethyl sulfoxide (DMSO) at 1 mg/ml for preparing solutions, then diluted to the appropriate concentrations with culture medium. On Day 3, the cells were washed with phosphate-buffered saline (PBS) twice and detached with trypsin (0.25%) plus EDTA (0.02%), and the resulting single-cell suspension was counted with a Coulter Counter Model ZBI (Coulter Electronics Inc, Hialeah, FL). The growth inhibition rate (GIR) was calculated as follows:

$$GIR\ (\%) = [1 - (Nt - No)/(Nc - No)] \times 100,$$

where Nt = the number of drug-treated cells on Day 3; No = the number of cells on Day 0; and Nc = the number of untreated control cells on Day 3. The 50% effective dose (ED50) was calculated from the dose-response curve by interpolation. In this condition, the effect of DMSO alone on cell growth was not observed.

Plasma Drug Concentration Assay

The mice were injected iv with 10 or 25 mg/kg of ECPT or CPT (0.1 ml/mouse). Then they were lightly anesthetized with ether and blood samples were collected by cardiac puncture with a heparinized syringe. Plasma was separated immediately after blood collection and stored at −20°C until assay. Drug was extracted from plasma by a modification of the method of Hart et al (21). In brief, the plasma (0.2 ml) was acidified with 1 ml of 0.1 N HCl, 3 ml of water-saturated ethyl acetate was added to the acidified plasma, and the mixture was centrifuged (3000 rpm, 10 mins). Two milliliters of the organic phase was transferred to a tube and the remainder (1 ml) was evaporated under a nitrogen stream at 40°C. The remaining aqueous phase was re-extracted with 3 ml of water-saturated ethyl acetate and 2 ml of the organic phase was transferred to the above tube. The combined organic phase (4 ml) was then evaporated and the residue was dissolved in 1 ml of methanol. A sample (20 μl) was applied on the high-performance liquid chromatograph (HPLC) system described later. The limit of the detection of plasma ECPT and CPT was about 0.03 μg/ml. In this experiment, at 12 hours drug concentrations of ECPT and CPT were observed as trace in records or showed large deviations and could not be calculated.

Mean values of the plasma concentration of ECPT and CPT were fitted by using the MULTI program (22). The algorithm of the nonlinear least-squares method was the Gauss-Newton method and the inverse square of the concentration was used as the weight to fit the curve for a wide range of drug concentrations. Biological half-life was calculated by using the curve-fitted data. The area under the curve (AUC) was calculated by the trapezoidal rule. Total-body clearance (CL) was calculated by the formula CL = dose/AUC.

Distribution Assay

Mice that were fasted overnight were injected iv with 25 mg/kg of ECPT (0.1 ml/mouse). Mice were given no food but allowed water ad libitum after the injection. After administration of the drug, the animals were lightly anesthetized with ether and blood was collected as described above. Liver, kidneys, and small and large intestines were excised and, except for the intestine, were rinsed with chilled saline, blotted on filter paper, and weighed. Contents of the large and small intestine were washed out with 2 and 5 ml of chilled distilled water, respectively. The washed intestine was rinsed with chilled saline, blotted on filter paper, and weighed. The plasma (0.2 ml) was diluted with 0.8 ml of water and acidified with three drops of concentrated HCl. Water-saturated ethyl acetate (5 ml) was added to the acidified plasma sample and extraction continued for 10 minutes. The mixture was then centrifuged (3000 rpm, 5 mins) and 1 ml of organic phase was evaporated under a nitrogen stream at 40°C. The residue was dissolved in methanol and a sample was applied on the HPLC system. Excised organs were placed in four volumes of chilled water and homogenized. To 1 g of homogenized mixture, three drops of concentrated HCl was added and extraction with 5 ml of water-saturated ethyl acetate continued for 10 minutes. The intestinal contents were sonicated and 1 ml of solution was ana-

lyzed by the same method as for plasma. The efficiency of extraction of ECPT from plasma and organs was 82% and 76%, respectively.

HPLC Operations and Assay Method

The HPLC (LC-4A, Shimadzu Seisakusho, Kyoto, Japan) was equipped with a fluorescence spectromonitor (RF-530, Shimadzu Seisakusho) and a 20-μl loop-injector (Rheodyne, Cotati, CA). The fluorescence spectromonitor was set at 379 and 440 nm for excitation and emission wavelengths, respectively. An octadecylsilanized silica column (15 × 0.46 cm, Shodex ODSpak F-411A, Showa Denko, Tokyo, Japan) was available. The mobile phase consisted of methanol and water (70/30, vol/vol, for ECPT and 60/40, vol/vol, for CPT). The flow rate was 1.0 ml/minute and the column temperature was kept at 40°C. Under the above conditions, the retention times of ECPT and CPT were 3.85 and 4.4 minutes, respectively.

Cell Cycle Progression Assay

Logarithmically growing L1210 cells (2×10^5/ml) in RPMI-1640 medium with 10% fetal bovine serum (GIBCO) were exposed to 100 ng/ml of ECPT or CPT for 24 hours (this concentration was enough to inhibit the cell growth completely). Then they were washed with PBS twice and fixed with 50% ethanol at 4°C for 30 minutes. For flow cytometry, cells were stained essentially as described by Krishan (23). The fixed cells were incubated in a 1-mg/ml solution of RNase in PBS for 30 minutes at 37°C. Subsequent centrifugation of the samples was followed by washing with distilled water and staining with propidium iodide solution (0.05 mg/ml in 0.1% sodium citrate) at 4°C for 30 minutes. The stained cells were analyzed in an FSC-1 cell sorter (Japan Spectroscopic, Tokyo, Japan).

RESULTS

Antitumor Activity Against L1210 Leukemia In Vivo

T/C values of drug-treated mice were calculated (table 1). Both drugs showed prolonged survival time at a total dose of 40–120 mg/kg through both administration routes. ECPT showed higher activity than CPT at total doses of 5, 10, and 20 mg/kg ip and 40 mg/kg iv. The administration of 120 mg/kg of both drugs showed toxicity, although injection of ECPT ip caused the highest T/C value. The weight changes of drug-treated and control mice were almost the same and no difference between the two groups of mice was observed. The acute toxicity of ECPT in ICR mice was lower than that of CPT; the LD50 values (mg/kg) were 239.9 (male) and 251.7 (female) for ECPT and 154.9 (male) and 173.8 (female) for CPT when the drugs were injected iv.

Inhibition of Growth of KB Cells In Vitro

KB cells cultured in vitro were exposed to the drugs and their growth inhibition rates were calculated (table 2; fig 2). ECPT and CPT had relatively low ED50 values (3.5 and 8.6 ng/ml, respectively), and the values were of the same order as that of MMC and lower than that of 5-FU. ECPT showed the lowest value among the four drugs and it was about twofold less than that of CPT. DMSO-dissolved ECPT and CPT were about twice as effective as their respective sodium salts.

TABLE 1.—Antitumor activity of ECPT and CPT against L1210 tumors*

Experiment No.	Sample	Total dose (mg/kg)	Survival time† (days, mean ± SD)	T/C (%)
1	None		8.3 ± 0.5	100
	ECPT	40 (13.3)	15.7 ± 0.5‡	189
		80 (26.7)	21.8 ± 3.5	263
		100 (33.3)	19.3 ± 5.2	232
		120 (40.0)	11.7 ± 9.4	141
	CPT	40 (13.3)	13.8 ± 2.5	166
		80 (26.7)	19.7 ± 3.5	237
		100 (33.3)	17.3 ± 5.8	208
		120 (40.0)	9.3 ± 2.1	112
2	None		8.1 ± 0.5	100
	ECPT	5 (1.7)	9.0 ± 0.6‡	111
		10 (3.3)	12.3 ± 0.7§	152
		20 (6.7)	13.7 ± 0.5§	169
		40 (13.3)	15.5 ± 3.2	191
		80 (26.7)	18.8 ± 6.1	232
		120 (40.0)	26.3 ± 8.9¶	317
	CPT	5 (1.7)	8.3 ± 0.5	102
		10 (3.3)	9.0 ± 0.6	111
		20 (6.7)	10.5 ± 0.8	130
		40 (13.3)	13.5 ± 2.3	167
		80 (26.7)	18.8 ± 3.2	232
		120 (40.0)	14.0 ± 5.8	173

*BDF_1 mice were inoculated with L1210 cells (5×10^5/mouse) ip on Day 0 and the drug was administered iv (Experiment 1) or ip (Experiment 2) on Days 1, 5, and 9 (10 mice/group). Values in parentheses are daily doses.

†No long-term survivor was observed.

‡Difference from corresponding CPT-treated mice, $P < 0.05$.

§Difference from corresponding CPT-treated mice, $P < 0.001$.

¶Difference from corresponding CPT-treated mice, $P < 0.01$.

Plasma Drug Concentration Analysis

As shown in figure 3, the concentration of ECPT or CPT in the plasma decreased rapidly shortly after ad-

TABLE 2.—Inhibition of growth of KB cells by ECPT and CPT in vitro

Drug	ED50 (ng/ml)
ECPT-Na*	3.5
ECPT-DMSO†	1.5
CPT-Na*	8.6
CPT-DMSO†	4.4
MMC	4.2
5-FU	23

*Drug was used as sodium salt.

†Drug was dissolved in DMSO at 1 mg/ml, then diluted to the appropriate concentration with culture medium. In this condition, no effect of DMSO alone on cell growth was observed.

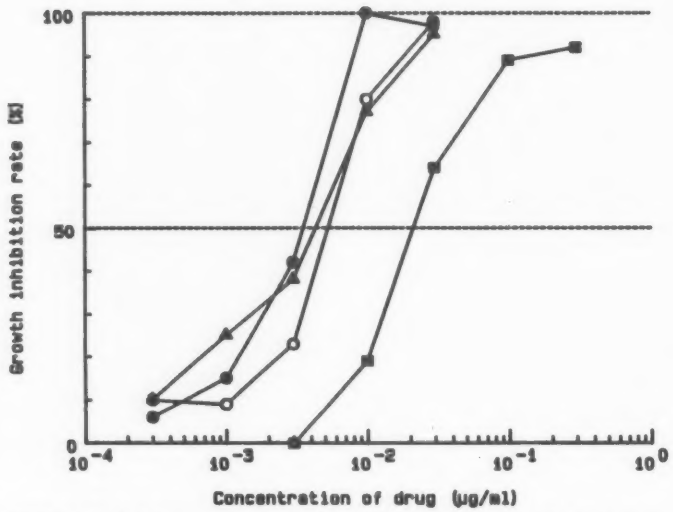

FIGURE 2.—Typical dose-response curves of drug-treated KB cells. KB cells in culture were exposed to the drug at various concentrations for 3 days, then the No. of cells/plate was determined and growth inhibition rates were calculated (*see* Materials and Methods section). ● = ECPT: ○ = CPT; ▲ = MMC; and ■ = 5-FU.

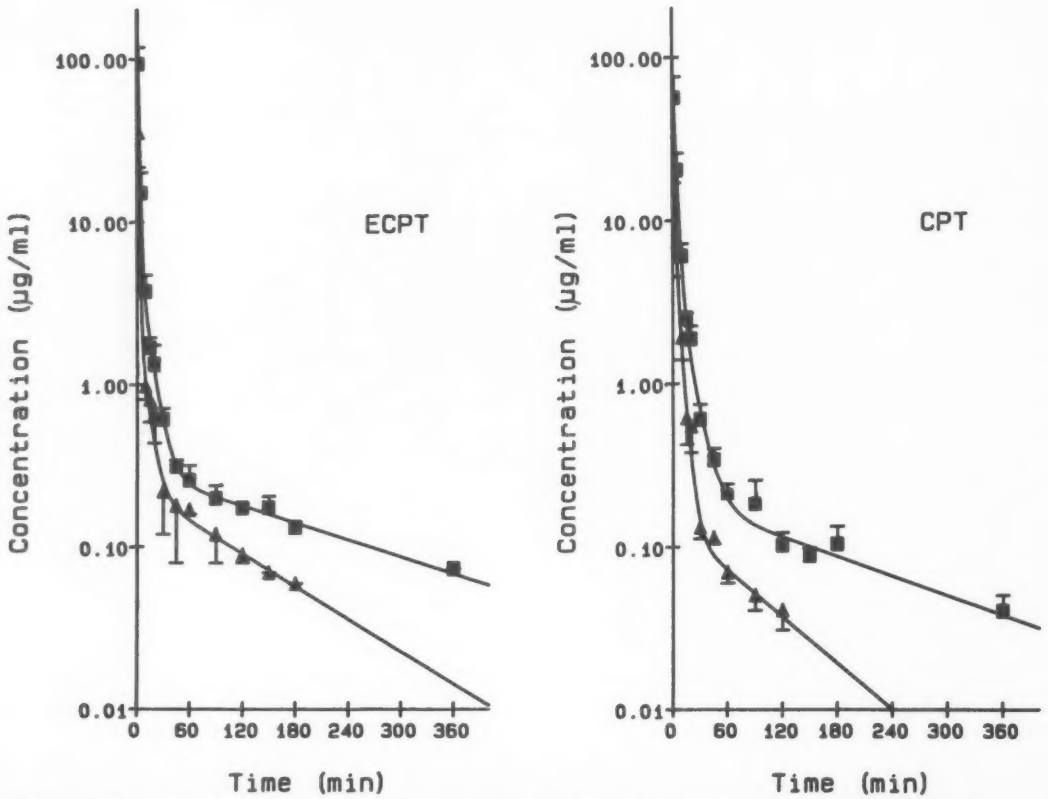

FIGURE 3.—Plasma concentration vs time curves of ECPT and CPT. CDF_1 mice were injected iv with 10 (▲) or 25 (■) mg/kg of ECPT or 10 (▲) or 25 (■) mg/kg of CPT. Points and bars are means ± SD for 4 mice at each period. Curves are from computer-fitted data.

TABLE 3.—Pharmacokinetic parameters of ECPT and CPT

Drug	Parameter	Dose (mg/kg) 10	25
ECPT	$\alpha_{t1/2}$ (mins)*	0.78 ± 0.13†	1.01 ± 0.09†
	$\beta_{t1/2}$ (mins)*	5.92 ± 1.60†	6.88 ± 0.66†
	$\gamma_{t1/2}$ (mins)*	90.31 ± 21.94†	173.25 ± 18.21†
	AUC (μg/min/ml)‡	149.52	406.50
	CL (L/kg/min)§	0.067	0.062
CPT	$\alpha_{t1/2}$ (mins)*	1.23 ± 0.66†	2.04 ± 0.39†
	$\beta_{t1/2}$ (mins)*	3.99 ± 1.29†	8.70 ± 1.92†
	$\gamma_{t1/2}$ (mins)*	63.41 ± 26.58†	151.46 ± 29.38†
	AUC (μg/min/ml)‡	84.06	332.06
	CL (L/kg/min)§	0.096	0.075

*$\alpha_{t1/2} = 0.693/\alpha$, $\beta_{t1/2} = 0.693/\beta$, $\gamma_{t1/2} = 0.693/\gamma$, where concentration = $A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-\gamma t}$ ($\alpha > \beta > \gamma$).
†Values for computer-fitted parameters ± SD.
‡AUC was calculated by the trapezoidal rule.
§CL = dose/AUC.

ministration and then slowly after 45 minutes. The mean plasma concentrations were determined by the nonlinear least-squares method and the values of the pharmacokinetic parameters calculated by the algorithm of the Gauss-Newton method are shown in table 3. Similar results were given by other algorithms. The biological half-life of the terminal phase of ECPT and CPT increased with the dose, and that of the initial phase of ECPT was shorter than that of CPT. However, the half-life of the terminal phase of ECPT was longer than that of CPT.

Drug Distribution

The levels of ECPT in the tissues after iv administration are shown in figure 4. The concentration of ECPT in plasma decreased rapidly in the same way as shown in figure 3 and the terminal phase decreased exponentially, with a half-life of about 2.5 hours. The concentration of the drug in the liver decreased slowly for the initial 30 minutes and then decreased rapidly for 2.5 hours; after that the decrease slowed down. The drug concentration of liver was larger than that of kidney at any time. The amount of drug in the small intestine was constant for the first 30 minutes after administration, decreased rapidly for the next 2.5 hours, and then decreased slowly until the end of the experiment (6 hours). The drug in the contents of the small intestine peaked at 30 minutes and then exponentially decreased until 6 hours. The level in the large intestine showed a peak at 3 hours and it increased from 30 minutes to 3 hours and then plateaued. The AUC for each organ or intes-

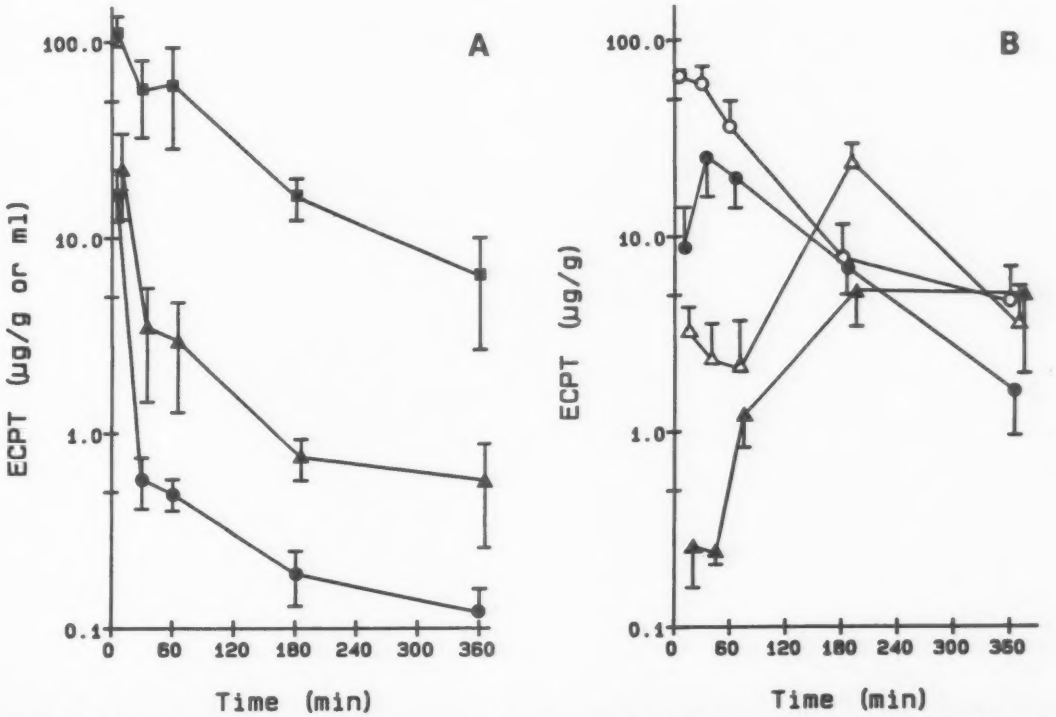

FIGURE 4.—Distribution of ECPT. CDF_1 female mice were injected iv with 25 mg/kg of ECPT. A: ● = plasma; ■ = liver; ▲ = kidney. B: ○ = small intestine; ● = contents of small intestine; △ = large intestine; ▲ = contents of large intestine. Values are means ± SD for 4 mice.

TABLE 4.—AUC for ECPT in various organs*

Time	Plasma (μg·hr/ml)	Liver (μg·hr/g)	Kidney (μg·hr/g)	Small intestine Wall (μg·hr/g)	Small intestine Contents (μg·hr)	Large intestine Wall (μg·hr/g)	Large intestine Contents (μg·hr)
0–15 mins	0.74	4.80	0.97	2.7	1.85	0.14	0.02
15–30 mins	3.70	35.83	5.59	25.88	35.95	1.16	0.21
30 mins–1 hr	0.27	29.54	1.62	23.73	56.94	1.11	0.73
1–3 hrs	0.68	77.40	3.37	43.45	134.10	25.90	13.02
3–6 hrs	0.47	33.83	1.98	18.35	63.68	40.83	30.87
0–6 hrs	5.84	181.40	13.89	114.12	292.52	69.14	44.85

*AUC was calculated according to the trapezoidal rule.

tinal contents showed that the drug accumulated in the intestinal tract (table 4).

Effect on Cell Cycle Progression In Vitro

The DNA histograms of L1210 exposed to ECPT and CPT in vitro were determined by flow cytometry (fig 5). The untreated control cells showed a DNA histogram with two main peaks of G_1 (about channel 90) or G_2-M (about channel 180), which is the typical pattern of the logarithmically growing cells. The histograms of ECPT- and CPT-treated cells showed one peak (about channel 180) which indicated G_2-M phase accumulation and an increase in small fluorescent cells. All cells treated with ECPT and almost all cells treated with CPT remained in the cell cycle from G_1 to G_2-M phase; cells in S phase were not observed.

DISCUSSION

In this study, CPT showed therapeutic efficacy against mouse L1210 leukemia similar to that reported elsewhere (1,2). ECPT showed higher antitumor activity against L1210 in vivo and growth inhibition activity against KB cells in vitro than did CPT. This suggests that ECPT itself has a higher potentiality to kill and/or inhibit the growth of tumor cells than does CPT. ECPT and CPT also inhibited tumor cell growth in vitro and DMSO-dissolved ECPT and CPT were more effective than their sodium salts. Similar results have been reported for the 3T3 and SV40-transformed 3T3 system in vitro (24). Since CPT is only slightly soluble in water, many studies have been carried out with its sodium salt. However, dissolving CPT in alkali, in which its lactone ring is opened, reduces its antitumor activity (25). This was also observed in in vitro and in vivo experiments

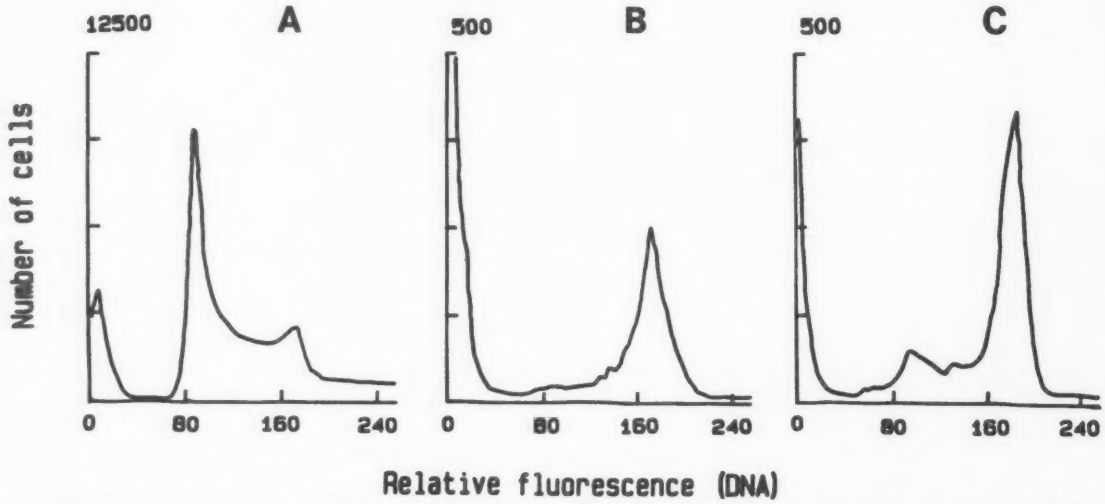

FIGURE 5.—Flow cytometry-determined DNA histograms of cultured L1210 cells treated with ECPT or CPT. Cells (2×10^5/ml) were exposed to the drug for 24 hrs at 37°C in growth medium, then harvested, stained for DNA analysis, and analyzed in a flow cytometer as described in the Materials and Methods section. A = untreated controls; B = ECPT, 100 ng/ml; C = CPT, 100 ng/ml.

with ECPT. In our previous studies (19,24), several derivatives of CPT with substitutions in the C_7 position were examined for various antitumor activities as well as growth inhibition. 7-Methylcamptothecin was more effective in vitro but highly toxic in vivo.

In the analysis of plasma concentration profiles of ECPT and CPT after iv administration, we found that concentration versus time curves were better expressed by the sum of three exponential terms, although Creaven and Allen (26) speculated that the decay of CPT could be changed from two to three exponentials by varying the degree of hydration during the experiment. We reached the conclusion that the triexponential expression was the best according to the critical value of AIC calculated by the fitting program (22); $AIC = n \times \log_e (SS) + 2m$, where n = number of data; SS = sum of squares of residual; and m = number of model parameters. The concentration of ECPT in plasma remained at a higher level and for a longer time than that of CPT, especially in the terminal phase after administration. This also explains the high activity in vivo, possibly because the tumor cells were exposed to a higher total dose of ECPT. The investigation on distribution of ECPT in mice showed an accumulation in the gastrointestinal tract, as is the case with CPT (16,27) and 10-hydroxycamptothecin (17,18). ECPT also remained at a high level in the intestinal tract. These findings suggest that the main route of excretion of the drugs is through the biliary tract.

DNA histograms of ECPT- and CPT-treated L1210 cells with accumulation of G_2-M phase suggest a G_2-M blocking effect of the drugs. Similar results have been obtained with CPT in human lymphocytes (2) and with 10-hydroxycamptothecin (18). Tobey (28) and Tobey and Crissman (29) have reported a delay in cell progression in synchronized Chinese hamster ovary cells treated with CPT. This suggests that the difference in sensitivity of cells to the drugs may be due to a difference in cycle progression of the cells.

In this study, distribution of ECPT in normal mice and the effects of the drug on cell cycle progression in vitro were similar to those of CPT. These results suggest that the main reasons for the superior antitumor activity of ECPT compared with CPT are that: (*a*) ECPT itself has stronger growth-inhibiting activity against tumor cells; and (*b*) it remains for a longer time and in a larger amount in the intestinal tract when administered in vivo.

REFERENCES

1. WALL ME, WANI MC, COOK CE, ET AL. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J Am Chem Soc 88:3888-3890, 1966.
2. GALLO RC, WHANG-PENG J, and ADAMSON RH. Studies on the antitumor activity, mechanism of action, and cell cycle effects of camptothecin. J Natl Cancer Inst 46:789-795, 1971.
3. LI LH, FRASER TJ, OLIN EJ, ET AL. Action of camptothecin on mammalian cells in culture. Cancer Res 32:2643-2650, 1972.
4. DREWINKO B, FREIREICH EJ, and GOTTLIEB JA. Lethal activity of camptothecin sodium on human lymphoma cells. Cancer Res 34:747-750, 1974.
5. JIANG T, SALMON SE, and LIU RM. Activity of camptothecin, harringtonine, cantharidin and curcumae in the human tumor stem cell assay. Eur J Cancer Clin Oncol 19:263-270, 1983.
6. BOSMANN HB. Camptothecin inhibits macromolecular synthesis in mammalian cells but not in isolated mitochondria or *E. coli*. Biochem Biophys Res Commun 41:1412-1420, 1970.
7. HORWITZ SB, CHANG C, and GROLLMAN AP. Studies on camptothecin. I. Effects on nucleic acid and protein synthesis. Mol Pharmacol 7:632-644, 1971.
8. KESSEL D. Effects of camptothecin on RNA synthesis in leukemia L1210 cells. Biochim Biophys Acta 246:225-232, 1971.
9. HORWITZ MS, and HORWITZ SB. Intracellular degradation of HeLa and adenovirus type 2 DNA induced by camptothecin. Cancer Res 31:1883-1887, 1971.
10. SPATARO A, and KESSEL D. Studies on camptothecin-induced degradation and apparent reaggregation of DNA from L1210 cells. Biochem Biophys Res Commun 48:643-648, 1971.
11. ABELSON HT, and PENMAN S. Induction of alkali labile links in cellular DNA by camptothecin. Biochem Biophys Res Commun 50:1048-1054, 1973.
12. GOTTLIEB JA, GUARINO AM, CALL JB, ET AL. Preliminary pharmacologic and clinical evaluation of camptothecin sodium (NSC-100880). Cancer Chemother Rep 54:461-470, 1970.
13. GOTTLIEB JA, and LUCE JK. Treatment of malignant melanoma with camptothecin (NSC-100880). Cancer Chemother Rep 56:103-105, 1972.
14. MUGGIA FM, CREAVEN PJ, HANSEN HH, ET AL. Phase I clinical trial of weekly and daily treatment with camptothecin (NSC-100880): correlation with preclinical studies. Cancer Chemother Rep 56:515-521, 1972.
15. MOERTEL CG, SCHUTT AJ, REITEMEIER RJ, ET AL. Phase II study of camptothecin (NSC-100880) in the treatment of advanced gastrointestinal cancer. Cancer Chemother Rep 56:95-101, 1972.
16. SCHAEPPI U, FLEISCHMAN RW, and COONEY DA. Toxicity of camptothecin (NSC-100880). Cancer Chemother Rep Part 3, vol 5(1):25-36, 1974.
17. YANG J, HAN J, and XU B. Distribution and excretion of 10-hydroxycamptothecin and its influence on immune response. Acta Pharmacol Sin 1:44-48, 1980.
18. CREASEY WA, RICHARDS M, GIL D, ET AL. Action of (*S*)-10-hydroxycamptothecin on P388 leukemia and distribution of the drug in mice. Cancer Treat Rep 67:179-182, 1983.
19. YOKOKURA T, SAWADA S, NOKATA K, ET AL. Antileukemic activity of new camptothecin derivatives. *In* Proceedings of the Japan Cancer Association, The 40th Annual Meeting, p 228, 1981.
20. MIYASAKA T, MUTAI M, SAWADA S, ET AL. Synthesis of new camptothecin derivatives. Jpn Kokai Tokkyo Koho, JP 56-158786:1981.
21. HART LG, CALL JB, and OLIVERIO VT. A fluorometric method for determination of camptothecin in plasma and urine. Cancer Chemother Rep 53:211-214, 1969.
22. YAMAOKA K, TANIGAWARA Y, NAKAGAWA T, ET AL. Pharmacokinetic analysis program (MULTI) for microcomputer. J Pharm Dyn 4:879-885, 1980.
23. KRISHAN A. Rapid flow cytometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 66:188-193, 1975.
24. NAGATA H, FUKADA M, NOTAKE K, ET AL. Effects of an antitumor alkaloid, camptothecin and its derivatives on cell growth of mammalian cells in culture. J Aichi Med Univ Assoc 11:286-293, 1983.
25. WANI MC, RONMAN PE, LINDLEY JT, ET AL. Plant antitumor agents. 18. Synthesis and biological activity of camptothecin analogues. J Med Chem 23:554-560, 1980.
26. CREAVEN PJ, and ALLEN LM. Renal clearance of camptothecin (NSC-100880): effect of urine volume. Cancer Chemother Rep 57: 175-184, 1973.
27. GUARINO AM, ANDERSON JB, STARKWEATHER DK, ET AL. Pharmacologic studies of camptothecin (NSC-100880): distribution, plasma protein binding, and biliary excretion. Cancer Chemother Rep 57:125-140, 1973.

28. TOBEY RA. Effects of cytosine arabinoside, daunomycin, mithramycin, azacytidine, and camptothecin on mammalian cell cycle traverse. Cancer Res 32:2720–2725, 1972.

29. TOBEY RA, and CRISSMAN HA. Use of flow microfluorometry in detailed analysis of effects of chemical agents on cell cycle progression. Cancer Res 32:2726–2732, 1972.

Phase I Trial of Caracemide Using Bolus and Infusion Schedules[1,2]

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We conducted a phase I trial of caracemide, a new chemotherapeutic agent, which is active in the MX1 (mammary) and CX1 (colon) human tumor xenografts. Using a 5-day bolus schedule, dose-limiting toxicity consisting of burning perioral pain associated with flushing, nasal stuffiness, and excess lacrimation was seen at 650 mg/m^2/day. Using a 5-day continuous-infusion schedule, dose-limiting toxicity in the form of changes in affect, lethargy, disorientation, and cognitive dysfunction with electroencephalogram abnormalities was noted at 800 mg/m^2/day. The recommended phase II dose levels are 525 mg/m^2/day using the 5-day bolus schedule and 650 mg/m^2/day using the continuous-infusion schedule. Because of venous pain at the site of infusion, the drug must be delivered via central venous access. The pathophysiology of both the peripheral and central side effects of caracemide may be related to increased cholinergic activity. [Cancer Treat Rep 71:349-352, 1987]

Caracemide is a new chemotherapeutic agent developed by the Dow Chemical Corporation. It is structurally unrelated to any current chemotherapeutic compound (fig 1). Preclinical testing revealed efficacy in MX1 (mammary) and CX1 (colon) human tumor xenografts, as well as P388 leukemia. However, it was ineffective in the following cell lines: B16 melanoma, CD8F mammary, Colon 38, Leukemia L1210, and Lewis lung tumors and the LX1 (lung) xenograft (1). Its mechanism of action is unknown, although it has been shown to be a modest inhibitor of ribonucleotide reductase (2). Animal toxicity included neurologic, gastrointestinal, renal, hepatic, and hematopoietic dysfunction which was dose- and schedule-dependent.

We conducted a phase I trial of caracemide using a bolus infusion schedule daily over 5 days. Because of what appeared to be dose-limiting peak level toxicity, we conducted a further study using a 5-day continuous-infusion schedule.

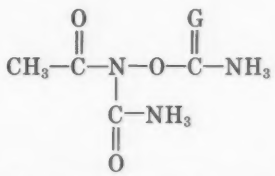

FIGURE 1.—Structure of caracemide.

METHODS AND RESULTS

Patients

Forty-two patients with histologic proof of malignancy were entered in this phase I trial. Characteristics of this group are shown in table 1. The majority of patients had received extensive prior therapy.

Bolus Schedule

The initial dose of caracemide given was 60 mg/m^2/day $\times$ 5 days administered over 30 minutes. This represents $\frac{1}{10}$ of the LD10 in mice. The dosage escalation schedule is shown in table 2. Using this schedule, 18 patients received 38 courses of therapy. There were three early deaths due to progressive disease. Toxicity (table 2) included pain at the site of iv infusion without objective signs of severe phlebitis, which necessitated drug delivery via a central line in all patients receiving doses $>$ 225 mg/m^2. One patient at 350 mg/m^2/day received part of the infusion through a peripheral iv. He was unable to tolerate this and completed therapy with a central line. Mild nausea and vomiting were noted at all dose levels. Mild burning perioral pain complicated six of seven courses of therapy at 525 mg/m^2. At 650 mg/m^2

[1] Received Oct 18, 1985; revised Nov 12, 1986; accepted Dec 12, 1986.

[2] Supported in part by contract N01-CM-27550 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

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TABLE 1.—Patient characteristics ($N = 42$)

Characteristic	No. of patients
Male/female	17/25
Median age in yrs (range): 54 (27–79)	
Performance status*	
0	5
1	25
2	9
3	3
Prior chemotherapy†	39
Prior radiation therapy	23
Tumor types	
Colon	13
Head and neck	5
Sarcoma	4
Melanoma	3
Lung	3
Cervix	3
Others	11

*Eastern Cooperative Oncology Group criteria.
†Median = 2 regimens; range = 0–8.

perioral pain was noted in nine of ten courses of therapy. In five patients it was considered severe. In three patients it was associated with flushing, nasal stuffiness, excess lacrimation, and a malar rash. This toxic effect necessitated cessation of therapy in one patient after 4 days of therapy and the refusal of two other patients to accept a second course at that dose level. Two of the seven patients treated at the 650-mg/m^2 dose level experienced thrombocytopenia, with normal wbc counts. Both patients died of progressive disease, with thrombocytopenia still present. One patient had deep venous thrombosis and a history of superior vena caval obstruction, and was being treated with heparin. He died on Day 13 of his second course of treatment, with a platelet count of 25,000/mm^3. The patient had been receiving heparin for 8 days. The second patient had no evidence of thrombotic phenomena or disseminated intravascular coagulation. She died on Day 15 of treatment with a platelet count of 92,000/mm^3. Neither patient was known to have bone marrow involvement. In the other patients treated, no hematologic toxicity was noted (table 2). There were no objective responses.

In an attempt to ameliorate the dose-limiting perioral burning sensation, patients were treated with Benadryl, hydrocortisone, and periactin without improvement. However, this symptom was somewhat ameliorated by prolonging the length of the infusion and by decreasing the concentration of the infusate. To determine if a bolus-infusion schedule was feasible we elected to study patients at the 650-mg/m^2 level with a 4-hour infusion in 1 L of 5% dextrose in water (approximately 1 mg/ml). Six courses of caracemide were given using this schedule; however, toxicity was still considered unacceptable. One patient received the drug over 12 hours without toxicity.

Continuous-Infusion Schedule

In an attempt to overcome the toxicity of the bolus schedule we undertook a second study giving caracemide as a continuous infusion through an indwelling central catheter over 5 days. The starting dose for this study was 350 mg/m^2/day. This dose was selected since it represented a reduction of two dose levels from the maximum tolerated dose established in the bolus study.

The stability of caracemide in dextrose and water is concentration-dependent. At a concentration of 2.7 mg/ml the $T9_0$ for caracemide is 5.7 hours.[4] Thus, all patients received the drug in 250-ml aliquots of 5% dextrose in water given 6 times daily over 4 hours.

Twenty-five patients received caracemide using the continuous-infusion schedule. One patient who had failed the bolus regimen was re-treated using the continuous-infusion schedule. The previously described toxic effect of burning perioral pain was seen only in one patient when the dose was incorrectly calculated at 800 mg/m^2 and infused over 8 hours (table 3). Mild nausea and vomiting were noted at doses $>$ 525 mg/m^2. Central nervous system (CNS) toxicity in the form of changes in affect, lethargy, disorientation, confusion, and cognitive dysfunction associated with electroencephalogram (EEG) abnormalities including diffuse slowing with occasional transient irritative activity in the temporal lobes was seen in four of six courses of therapy at 1200 mg/m^2 (table 4) (3). Three patients treated at that dose died of progressive disease 2–3 months after completing treatment. At the time of death the acute confusional state experienced by these patients had improved

TABLE 2.—Dose escalation: bolus schedule

Dose level (mg/m^2/day)	No. of—		No. of courses associated with Grade 3 or 4 World Health Organization toxicity					
	Courses	Patients	Nausea, vomiting, diarrhea	Venous pain	CNS	Perioral burning and flushing	Neutropenia	Thrombocytopenia
60	4	3	—	—	—	—	—	—
100	4	4	—	2	—	—	—	—
150	3	3	—	1	—	—	—	—
225	5	5	—	—	—	—	—	—
350	5	5	—	1	—	—	—	—
525	7	4	—	—	—	1	—	—
650	10	7	1	1	1	5	—	1

TABLE 3.—Dose escalation: infusion schedule

Dose level (mg/m^2/day)	No. of—		No. of courses associated with Grade 3 or 4 World Health Organization toxicity					
	Courses	Patients	Nausea, vomiting, diarrhea	Venous pain	CNS	Perioral burning and flushing	Neutropenia	Thrombocytopenia
350	4	3	—	—	—	—	—	—
525	3	3	—	—	—	—	—	—
650	16	12	—	—	1	—	1	—
800	10	9	—	1	2	1	1	1
1200	6	5	—	—	3	—	—	—

significantly, but they had not completely recovered. The other patients recovered completely.

Because CNS toxicity was not seen in the first three courses of caracemide given at 800 mg/m^2, we prospectively analyzed seven further courses at that dose. All patients received pretreatment neuropsychiatric evaluation including an EEG and computerized tomographic scan of the brain. They were followed with daily tests of cognitive function and repeat EEGs. Four of these seven courses were complicated by neurotoxicity. In two patients the drug was discontinued and both patients recovered within 48–72 hours. Two patients with Grade 2 toxicity recovered promptly. In one patient the drug was cautiously continued for 24 hours after symptoms appeared and the patient developed severe confusion, disorientation, paranoid ideation, and suicidal behavior. Her mental status returned to normal by Day 17. Eleven courses of therapy were given at 650 mg/m^2 with prospective neuropsychiatric evaluation. Two patients who could not complete treatment with 800 mg/m^2 because of CNS toxicity were successfully treated at 650 mg/m^2. Of the 11 courses, one was complicated by confusion, lethargy, and disorientation. Three courses were associated with mild transient changes in affect and lethargy (table 4). One patient with brain metastases and one with an abnormal pretreatment neuropsychiatric evaluation received caracemide at 650 mg/m^2. Neither patient developed neurotoxicity, and in the prospectively studied patients no predisposing factors for neurotoxicity were noted. There was no association between age and neurotoxicity. All patients with neurotoxicity had adequate renal and hepatic functions and performance status < 2 (Eastern Cooperative Oncology Group). There was no evidence of cumulative toxicity in the four patients who received multiple courses of therapy.

TABLE 4.—Neurotoxicity of caracemide using infusion schedule

Dose level (mg/m^2/day)	No. of courses*	Grade—			
		1	2	3	4
350	4	—	—	—	—
525	3	—	—	—	—
650	16 (11)	3	—	1	—
800	10 (7)	—	2	2	—
1200	6 (1)	1	—	—	3

*Nos. in parentheses refer to those courses prospectively evaluated.

In the majority of courses in which significant neurotoxicity was noted, symptoms appeared during the infusion on Day 3, 4, or 5. One patient developed symptoms on Day 8, although her EEG on Day 5 was abnormal. Prospective neuropsychiatric evaluation of patients provided a clinical picture of lethargy and alterations in affect, proceeding to agitation with confusion and disorientation, and finally resulting in global cognitive impairment associated with EEG changes. Short-term verbal and visual memory and visuospatial orientation appeared to be the higher mental functions most sensitive to caracemide and were generally the last to recover. At high doses, severe and persistent dementia occurred. Most patients improved within 2–3 days after discontinuation of the drug, although at the highest dose level, improvement was considerably slower and incomplete as mentioned above. Significant hematologic toxicity was not seen using the infusion schedule. One patient with colon cancer had a transient objective response in her metastatic liver disease as measured by ultrasound (less than a partial remission).

CONCLUSIONS

Caracemide is a new chemotherapeutic agent. Using a daily bolus-infusion schedule the drug must be given via a central venous catheter to avoid venous pain at the site of infusion. The dose-limiting toxic effect is burning perioral pain associated with flushing, excess lacrimation, and malar rash when caracemide is given over 4 hours at approximately 1 mg/ml. Based on this study the phase II starting dose should be 525 $mg/m^2/day \times 5$.

When caracemide is given using a 5-day continuous-infusion schedule, dose-limiting toxicity is neurologic dysfunction including lethargy and changes in affect, proceeding to confusion and disorientation associated with EEG abnormalities. The neurotoxicity observed during this study, which is compatible with central cholinergic overdrive, may be explained by the formation of methyl isocyanate, a known potent inhibitor of anticholinesterase activity, as a byproduct of caracemide degradation in plasma (4). The recommended dose for phase II trials of caracemide using a 5-day continuous schedule is 650 $mg/m^2/day$. Cognitive function must be evaluated during therapy, and if abnormalities develop in behavior, cognition, or affect the drug should be discontinued. If neurotoxicity is indeed caused by increased

cholinergic activity, it is possible that treatment with anticholinergic drugs may be helpful.

REFERENCES

1. CLINICAL BROCHURE. Caracemide (NSC-253272). Bethesda, MD, National Cancer Institute, 1983.
2. MOORE EC, and LOO TL. Inhibition of ribonucleotide reductase by caracemide. Cancer Treat Rep 68:1293–1296, 1984.
3. ADAMS F, KAVANAGH J, RABER M, ET AL. Neuropsychiatric manifestations of caracemide. Proc ASCO 50, 1985.
4. NEWMAN RA, FARQUHAR D, LU K, ET AL. Biochemical pharmacology of *N*-acetyl-*N* (methylcarbamoyloxy)-*N'* methylurea (caracemide). Biochem Pharmacol, in press.

Verapamil Reversal of Vincristine Resistance and Cross-Resistance Patterns of Vincristine-Resistant Chinese Hamster Ovary Cells[1,2,3]

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The phenotypic properties of a large number of low-level vincristine (VCR)-resistant Chinese hamster ovary cell lines have been studied. In particular, we examined the correlation between the reversal of VCR resistance by verapamil (VRP) and cross-resistance patterns to other drugs. Cross resistance to other vinca alkaloids developed in parallel with VCR resistance. Doxorubicin resistance was observed in many of the cell lines, occurring even at the lowest levels of VCR resistance. Increased sensitivity to taxol (TAX) was a feature of half of the lines tested and there was no increased resistance to 5-fluorouracil or chlorambucil. Two main groups of mutants have been identified: (*a*) those that are thought to be membrane mutants which are cross-resistant to TAX and whose VCR resistance is reversible by VRP; and (*b*) those that appear to be tubulin mutants, which have increased sensitivity to TAX and resistance to VCR in the presence of VRP. It was not possible to distinguish between the different mechanisms of low-level resistance by drug accumulation studies in unsupplemented medium. However, VCR accumulation in the presence of VRP did correlate with the cross-resistance results. [Cancer Treat Rep 71:353-359, 1987]

The development of resistance to the antimicrotubular anticancer drug vincristine (VCR) is a major problem during its clinical use. Understanding the phenomenon may contribute to the design of effective combination chemotherapy schedules which avoid cross resistance to other drugs and to the development of more effective related drugs.

VCR-resistant cell lines, both those established from experimental tumors (1) and those selected in vitro, are commonly found to have decreased drug accumulation within the cell (2-5). It has been suggested that resistance in these cases is caused, at least in part, by a change in the membrane leading to impaired accumulation due to increased efflux of the drug. However, these studies do not exclude the possibility of resistance caused by a change in the structure of the microtubular components, which would lead to reduced binding of VCR to its target site on the tubulin, and therefore decreased accumulation.

There is evidence from cross-resistance studies that both the above mechanisms operate but at different levels of VCR resistance (6). High levels of VCR resistance (> 50-fold) usually simultaneously confer some resistance to unrelated drugs [eg, doxorubicin (7)], suggesting that in these cells the membrane is the major site of resistance. The reverse is also true; cells selected for high levels of resistance to other drugs are often cross-resistant to VCR (8-10).

With lower levels of VCR resistance the mechanism is not so clear. Low levels of resistance do not uniformly confer cross resistance to other classes of drug (6), suggesting that in these cells, changes in tubulin binding may be the major mechanism of resistance. Cell lines selected for low-level resistance to demecolcine have been shown to have changes in the tubulin by 2D gel electrophoresis and demecolcine binding assays (11-13).

Since low levels of resistance can cause the failure of chemotherapy, it is important to clarify the mechanisms of such low-level resistance and the relative frequencies of the different classes. We have therefore examined the phenotypic properties of a large number of Chinese hamster ovary (CHO) cell lines selected for low levels (twofold to 16-fold) of VCR resistance. Although we must be cautious about relating work in such cell cultures to the in vivo situation, this type of study has value in that it can show the types of variant cross-resistance patterns which are possible.

We have used our data on the phenotypic properties of

[1] Received June 17, 1986; revised Nov 20, 1986; accepted Dec 12, 1986.
[2] Supported by the Yorkshire Cancer Research Campaign.
[3] Vinca alkaloids were donated by E. Lilly and Co, and taxol was supplied by the National Cancer Institute, Bethesda, MD.
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[5] We thank Mrs. M. Nicholls and Miss J. Ferguson for their excellent technical assistance.
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our low-level VCR-resistant mutants to consider the following questions.

(*a*) Is it possible to identify mutants with resistance to one of the vinca alkaloids but not to another?

(*b*) Are there any drugs which are consistently effective against VCR-resistant cell lines?

(*c*) Does resistance to VCR confer hypersensitivity to other agents? Earlier work has shown that some cell lines which are resistant to VCR have collateral sensitivity to taxol (TAX) (14). TAX is an antitumor agent which is at present on trial, and it is unusual among the antimicrotubular drugs in that it stabilizes rather than destabilizes microtubules (15). If a substantial proportion of VCR-resistant cell lines show collateral sensitivity, this could be of interest in designing effective chemotherapy schedules.

(*d*) Tsuruo (1) has shown that calcium channel blockers such as verapamil (VRP) can reverse some cases of VCR resistance. Is VRP reversal of VCR resistance a widespread phenomenon in cell lines with low levels of VCR resistance?

(*e*) To what extent is the accumulation of VCR in the presence and absence of VRP correlated with the cross-resistance patterns?

MATERIALS AND METHODS

Cell Culture Techniques

Cells were grown as previously described (14). Wild-type cells were E29pro+, provided by Dr. V. Ling of the Ontario Cancer Institute.

VCR-resistant sublines were selected in 24-well dishes. Cells (10^5/well) were plated and left to attach for 24 hours. Following UV irradiation to increase mutation frequency, the cells were kept in the dark. After 2 days fresh medium containing 0.125 μg/ml of VCR (4 × the D10 value for unselected cells) was added to the plates, and the medium was changed whenever necessary. One to three resistant colonies were observed in each well after 28 days and one colony was cloned off from each well for further study. Picking only one mutant from each well meant that the cell lines obtained were of independent origin.

The 71 resistant cell lines obtained were tested for levels of resistance to VCR and to a variety of other clinically used drugs using two techniques. Ten of the 71 lines were studied in detail. Their viabilities were tested in 24-well dishes (Nunc). Cells were plated at a density of 100 cells/well in various concentrations of the drugs. After 1 week cells were stained with Leishmans, and colonies of > 50 cells were counted. A value for relative resistance compared with unselected control cells could then be calculated as previously mentioned (14).

In view of the large number of mutants involved, a rapid test of resistance was conducted using 96-well plates for the remaining 61 mutants. For each drug, the highest concentration required was plated, and serial dilutions were then made across the plate leaving a control with drug-free medium only.

Cells to be tested were then added at a density of 50/well. After a 1-week growth, the cells were stained with Leishmans and checked for growth on each concentration. Data were collected on a growth/no growth basis and cell lines could be classified into groups using this technique. The results were similar to those obtained using the 24-well dishes.

^{3}H-VCR Accumulation Studies

Cells (4×10^5) of each of the ten lines were seeded into wells of a 24-well plate and incubated for 16 hours. The medium was then replaced with Hanks' balanced salt solution containing 0.1 μg/ml of ^{3}H-VCR (specific activity, 5.8 mCi/mol). Triplicate wells were exposed to drug for 5, 10, 20, and 30 minutes. The Hanks' solution was removed and the cells were trypsinized with ice-cold trypsin, placed in cold medium, and spun at 900 rpm in a refrigerated bench-top centrifuge for 10 minutes. The supernatant was poured off and cold isotonic saline was added. The cells were pelleted and finally resuspended in 1 ml of saline. Two-hundred microliters was removed for determination of cell number. The remainder of the cells were lysed with sodium dodecyl sulfate (1% final) prior to scintillation counting.

Drugs

VCR, vinblastine (VLB), and vindesine (VDS) were gifts from E. Lilly and Co; TAX was a gift from Dr. J. Douros of the National Cancer Institute; 5-fluorouracil (5-FU), doxorubicin (ADR), chlorambucil (CHL), and VRP were from Sigma; and ^{3}H-VCR was from Amersham.

RESULTS

Cross-Resistance Studies

Seventy-one VCR-resistant cell lines were selected. Under the test conditions used, control cells were not viable at VCR concentrations > 0.07 μg/ml. The relative resistance of each mutant as compared with the sensitive cell line was calculated and the distribution of resistance is shown in figure 1.

The cross-resistance patterns of ten mutants were studied in detail and those of the remaining 61 mutants by a more rapid screening method as outlined in the Materials and Methods section.

The mutants were tested for cross resistance to other vinca alkaloids, VLB and VDS, and to the functionally unrelated drugs ADR, 5-FU, CHL, and TAX. The results are shown in table 1. Resistance is relative to D10 values of control cells, which were VLB, 0.02 μg/ml; VDS, 0.1 μl/ml; ADR, 0.24 μg/ml; and TAX, 0.11 μg/ml. Values of relative resistance > 1 indicate resistance, while values < 1 indicate greater sensitivity than that of control cells.

There is clearly a very strong correlation between the level of VCR resistance and VLB or VDS resistance (correlation coefficients, 0.98 and 0.96, respectively). The

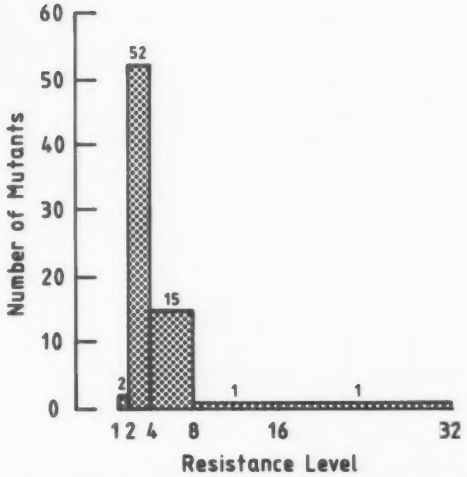

FIGURE 1.—Distribution of levels of resistance to VCR among 71 independently isolated VCR-resistant CHO cell lines, expressed relative to the resistance of control cells (E29).

61 other mutants tested by the rapid method followed a similar pattern.

There is a strong correlation between VCR and ADR resistance (correlation coefficient, 0.97). Of the other mutants tested by the rapid method, 40 showed no significant increase in resistance to ADR. However, due to the nature of the test, it is possible that levels of resistance of less than twofold exist. ADR cross resistance is not a phenomenon confined to higher levels of VCR resistance. It occurs at very low levels in parallel with the development of resistance to VCR.

None of the ten mutants under particular study or any of the 61 tested by the rapid method showed any increase in resistance to 5-FU. Many of the lines showed slightly increased sensitivity to this drug. There was also no increased resistance to CHL in the ten mutants tested (data not shown).

TABLE 1.—Resistance of VCR-resistant lines to VLB, VDS, ADR, and TAX relative to that of control cells

Resistant cell line	Relative resistance to—				
	VCR	VLB	VDS	ADR	TAX
B4	1.9	1.5	1.6	1.7	2.9
B35	2.1	2.2	2.6	1.2	0.4
B16	2.3	1.9	2.1	1.5	2.6
B24	2.4	2.3	2.1	1.4	0.4
B74	2.4	2.5	2.8	1.3	1.7
B68	3.0	2.3	—	1.6	0.8
B80	3.7	4.1	5.2	2.1	2.4
B87	4.0	4.3	4.0	3.5	3.0
B44	6.5	5.8	6.3	5.7	3.4
B38	16.0	14.3	9.9	16.9	6.6

Two of the ten mutants (B35 and B24) showed increased sensitivity to TAX. The other eight lines showed an overall trend of increased TAX resistance with increased VCR resistance (correlation coefficient, 0.84), suggesting that relatively high levels of VCR resistance tend to confer TAX resistance. Of a total of 36 lines tested, 18 showed increased sensitivity to this drug. This sensitivity was not restricted to the cell lines with the lowest levels of drug resistance, and cross resistance to TAX can be obtained in cell lines with very low levels of VCR resistance.

VRP Reversal of VCR Resistance

The VCR resistance of the ten cell lines was examined in the presence of 3 μg/ml of VRP, a concentration similar to that used by Tsuruo (1). The results are shown in table 2.

VRP reduced the resistance of wild-type cells to 11% of the control value. Although some effect of VRP on VCR resistance of control cells has been observed by Tsuruo (1), this reduction is greater than is usually found for control cells. It can be seen from the table that VRP increased the VCR toxicity even more in some of the resistant cell lines. As a general trend, the more highly resistant the cell line, the greater the reduction in resistance caused by VRP (correlation coefficient, −0.76). This trend has been reported previously by Tsuruo (1).

A novel and interesting second observation emerges from the data. Only two of the VCR-resistant cell lines (B35 and B24) maintained their relative levels of resistance to VCR in the presence of VRP. The VCR D10 values of E29 with or without VRP were 0.004 or 0.04 μg/ml, respectively, whereas the corresponding values for both B35 and B24 were 0.01 or 0.1 μg/ml, respectively. These are the same two lines which showed significantly increased sensitivity to TAX (table 1). In con-

TABLE 2.—Reversal of VCR resistance of resistant cell lines in the presence of 3 μg/ml of VRP*

Cell line	Relative resistance to VCR alone	Relative resistance to VCR + VRP	Resistance to VCR + VRP/ resistance to VCR − VRP	Proportion of resistance maintained in VRP relative to control
E29	[1.0]†	[1.0]†	0.11	[1.0]†
B4	1.9	1.2	0.07	0.63
B35	2.1	2.1	0.10	0.98
B16	2.3	1.7	0.08	0.75
B24	2.4	2.2	0.10	0.92
B74	2.4	1.8	0.08	0.75
B68	3.0	2.1	0.08	0.71
B80	3.7	1.5	0.04	0.41
B87	4.0	2.5	0.07	0.62
B44	6.5	2.5	0.04	0.38
B38	16.0	3.2	0.02	0.20

*Resistance levels are expressed relative to that of unselected control cells (E29) in Columns 1 and 2. Increase in sensitivity effected by VRP for each cell line is given in Column 3 and these values are normalized with respect to control cells in Column 4.

†Values arbitrarily set to 1.0.

trast, all of the cell lines that were resistant to TAX had their VCR resistance reversed to some extent by VRP.

^{3}H-VCR Accumulation Studies

Accumulation of ^{3}H-VCR was tested in the ten cell lines using 0.1 μg/ml of ^{3}H-VCR in the absence and the presence of 3 μg/ml of VRP. Preliminary experiments had shown that the rate of accumulation of ^{3}H-VCR was proportional to external concentration up to the highest concentration tested (5 μg/ml), suggesting passive accumulation. The results were analyzed statistically comparing the gradients of accumulation for each cell line with those for E29 using analysis of variance with test for linearity according to Armitage (16). Graphical results for representative cell lines B4, B35, B38, and B24 versus E29 are shown in figure 2, and the overall results are shown in table 3.

At the lowest levels of VCR resistance, VCR accumulation was not significantly different from that of control cells for mutants of either cross-resistance pheno-

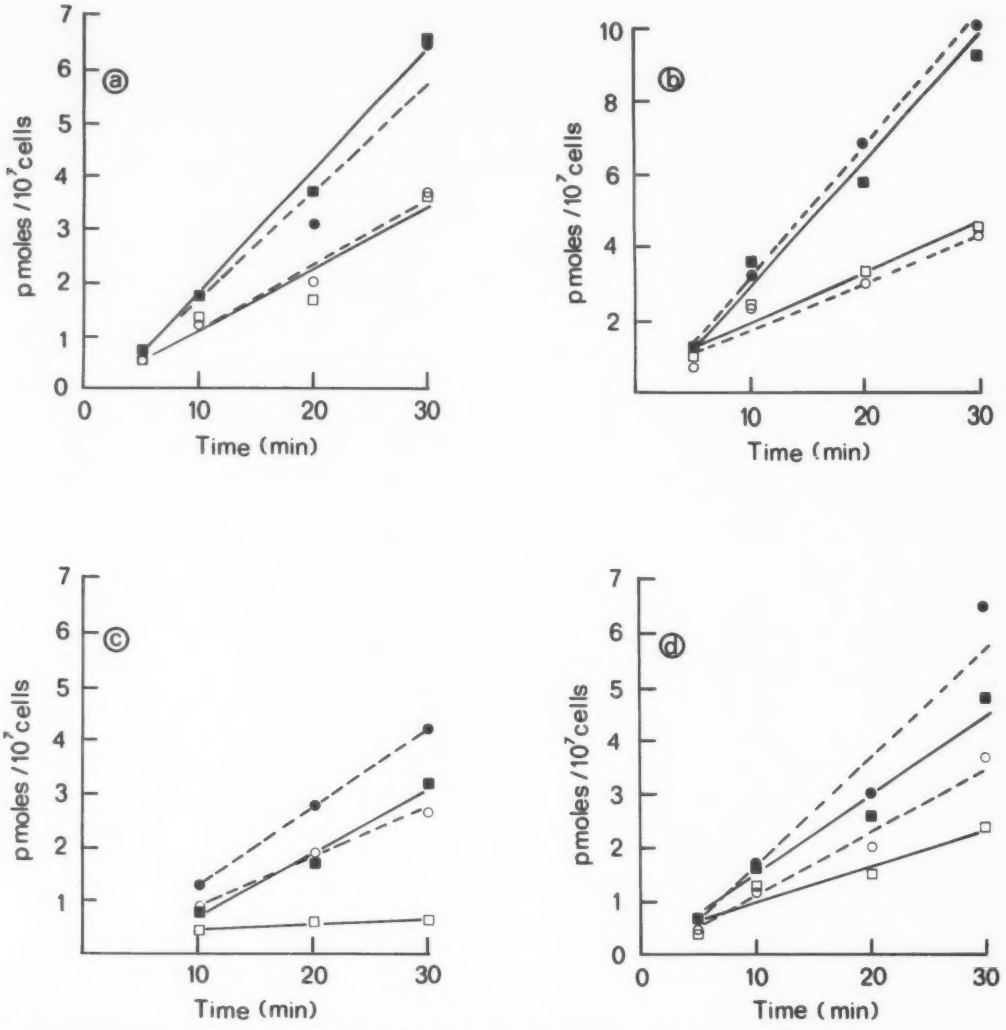

FIGURE 2.—VCR accumulation by 3 VCR-resistant mutants in the absence and in the presence of 4 μg/ml of VRP compared with VCR accumulation by control cells (E29). (*a*) B4 and E29, (*b*) B35 and E29, (*c*) B38 and E29, and (*d*) B24 and E29. The following symbols have been used throughout: □–□ = resistant mutant, without VRP; ■–■ = resistant mutant, with VRP; ○–○ = E29, without VRP; and ●–● = E29, with VRP. Maximum SE of any point was 0.40 pmol/10^7 cells; mean SE was 0.12 pmol/10^7 cells.

TABLE 3.—Reduced accumulation of ^{3}H-VCR by the resistant cell lines in the absence and in the presence of 3 μg/ml of VRP

Cell line	Reduced accumulation	VRP reversal of reduced accumulation*
B4	No	NA
B35	No	NA
B16	No	NA
B24	Yes†	No‡
B74	Yes‡	Yes
B68	No	NA
B80	Yes§	Yes
B87	Yes†	Yes
B44	Yes‡	Yes
B38	Yes†	Yes

*NA = not applicable.
†0.1% level of significance.
‡1% level of significance.
§5% level of significance.

type (fig 2, a and b). The similarity between mutants and control is seen in the absence or the presence of VRP.

At higher levels of VCR resistance, reduced VCR accumulation is usually observed (table 3). The resistant mutants with reduced VCR accumulation can, however, be subdivided on the basis of the effect of VRP on the reduced rates of accumulation. In five cell lines (B74, B80, B87, B44, and B38) VRP increased the rate of accumulation up to that of control cells (E29) (fig 2c). Thus, the rate of accumulation in E29 was increased 1.5-fold by VRP, but the rates in the five mutants were all increased by several times this figure. These five lines are cross-resistant to TAX, have increased sensitivity to VCR in the presence of VRP in clonogenic assays (above), and are thought to be membrane mutants. In contrast with the behavior of these five lines, B24 showed reduced accumulation which was only increased by a similar ratio (approximately 1.5-fold) as the control (fig 2d) and did not rise to the same rate as the control cells in VRP (significant difference at the 1% level). This cell line is hypersensitive to TAX, its VCR resistance as measured in clonogenic assays is not abolished by VRP, and it is thought to be a microtubule mutant.

Therefore, VCR accumulation in unsupplemented medium does not appear to give a clear indication of the type of mutant at low levels of resistance. However, if reduced accumulation is observed, the addition of VRP to the medium may distinguish the underlying mechanisms of resistance which give rise to the different cross-resistance patterns.

DISCUSSION

Our analysis of the phenotypic properties of low-level VCR-resistant mutants has revealed certain properties which are consistent among all of the mutants tested and other properties which enable subgroups to be identified. The properties which were common to all of the mutants studied were cross resistance to VLB, VDS, and ADR and lack of cross resistance to 5-FU and CHL. The properties which enable subdivision of the mutants include TAX resistance/increased sensitivity, VRP reversal of VCR resistance, and VRP reversal of reduced VCR accumulation.

The observation that low levels of VCR resistance confer cross resistance to VLB and VDS is in agreement with results of others (2,17,18), and it is likely that the lack of cross resistance reported by some investigators in the clinical situation (19,20) is mainly due to differences in regimen. For example, resistance to VCR is often noted when it is given at low concentrations in combination with other drugs, and high-dose VDS may then be administered as a single agent and prove to be effective.

The cell lines showed no resistance to 5-FU. These results agree with those of Ling (21) and are to be expected, since a mutation which confers resistance to VCR by a tubulin change would not affect the functioning of 5-FU and a mutation conferring resistance to VCR by a change in the membrane would be unlikely to affect uptake of a small molecule like 5-FU. However, these results differ slightly from those of Gupta (17), who found that a VLB-resistant CHO cell line with a very specific pattern of resistance only to some microtubule inhibitors showed enhanced sensitivity to the drug, whereas a second line which was thought to be a permeability mutant showed increased resistance to 5-FU. The reason for this discrepancy is not clear.

There was also no increased resistance to CHL in the mutants. This pattern of cross resistance has been less well-documented, but Gupta (17) found no change in CHL resistance in VLB-resistant CHO cells and Ling (21) found that lines selected for resistance to colchicine were hypersensitive to cyclophosphamide, another alkylating agent. These results are not so easy to explain. Obviously, a mutation causing resistance to VCR via a tubulin change would not confer resistance to CHL, whereas a change in the membrane may reduce the permeability of CHL, since CHL is not a very small molecule. It is, however, considerably smaller than VCR and may be taken up via a different mechanism.

Many of the cell lines were resistant to ADR. In previous studies ADR cross resistance has often been used as evidence of a membrane change and is usually only reported in cell lines with relatively high levels of resistance (7,22). However, very few studies have investigated the effect of ADR on cells with low levels of VCR resistance. Conter and Beck (6) showed that pleiotropic cross-resistance patterns occurred at high levels of VCR resistance, while at low levels the resistance was more specific, but it still included resistance to ADR. This study confirms and extends their results that even at very low levels of VCR resistance, concurrent ADR resistance is possible. Since some of the cell lines with ADR resistance also showed increased sensitivity to TAX, it seems unlikely that the only possible mechanism of ADR cross resistance is a membrane change conferring pleiotropic cross resistance to a large number

of drugs. It may be that the primary mechanism of action of ADR is related to that of VCR in some way, since it is becoming apparent that other mechanisms apart from DNA intercalation may be important in the cytotoxic action of ADR (23). The other possibility is that all ten lines tested in greater detail had pleiotropic cross-resistance patterns, perhaps a membrane change causing resistance to unrelated drugs such as ADR, but that in some cases, this caused TAX sensitivity at the level of the membrane. It has recently been shown (25,26) that the high-molecular-weight membrane glycoprotein whose presence correlates with multidrug resistance specifically binds vinca alkaloids in KB cells and hamster lung cells. It would obviously be of interest to investigate its binding to other drugs.

One-half of the cell lines tested showed increased sensitivity to TAX. This is in agreement with our earlier work (14) which showed that some cell lines selected for resistance to other antitubulin drugs were hypersensitive to TAX. This may be useful for rescue therapy but it has to be remembered that a significant proportion of lines show cross resistance to this drug. These results suggest two main classes of variants. We speculate that those which are resistant to TAX may be membrane mutants, whereas those which are hypersensitive to TAX may have some change either in the structure of the tubulin or in its lability causing them to be resistant to VCR and show increased sensitivity to TAX. Gupta (17) found that mutants resistant to VLB fell into two classes: one class had a specific resistance pattern to only some antitubulin drugs and the site of change was thought to be in the tubulin; and the other class showed cross resistance to a variety of nonrelated drugs and the site of change in this group was thought to be at the membrane level.

Our data on reversal of VCR cytotoxicity by VRP are broadly in agreement with those of Tsuruo et al (24). In general, the more resistant the cell line, the greater the effect of VRP. Our results do differ in several respects though: Tsuruo (1) obtained complete reversal of VCR resistance in the tumor cells used. In all cases, we only obtained partial reversal of the resistance. This may be due to differences in the cell lines used or to the fact that Tsuruo's lines were highly resistant. The other difference is that we obtained some lines which were not responsive at all to the potentiating effects of VRP. Resistant cell lines of this class may be obtained only during selection for low levels of resistance such as those employed here.

An inverse relationship exists between resistance to TAX and resistance to VCR in the presence of VRP (apart from B68 which is borderline in sensitivity to TAX). Cell lines which showed increased sensitivity to TAX were resistant to VCR in the presence of VRP and vice versa. This suggests that VRP can overcome the effects of a membrane change which confers cross resistance to unrelated drugs. It cannot, however, overcome resistance in lines which show a more specific pattern of cross resistance which we speculate may be due to a tubulin change. Such mutants are likely to be hypersensitive to TAX. This reciprocal relationship may be exploitable in the clinical situation. One mechanism of resistance to VCR is likely to be circumvented by VRP, and the other is likely to lead to increased sensitivity to TAX.

It would be helpful to have a quick method which could distinguish the types of mutant and could therefore predict the cross-resistance patterns of each line. At the very lowest levels of resistance tested, the two types of mutant were not distinguishable, since mutants of either type showed no detectable change in drug accumulation. This is probably due to the fact that the relationship between accumulation and drug resistance is not linear. At higher levels of resistance, detectable impaired accumulation occurred in six mutants; five were cell lines showing a pleiotropic cross-resistance pattern, including cross resistance to TAX, and thought to be membrane mutants. The sixth mutant, which showed reduced accumulation, was one which would not be expected to have impaired uptake since it is hypersensitive to TAX. Our speculation is that B24 is a tubulin mutant and there is less bound VCR in the cell, and therefore lower accumulation. VCR accumulation, therefore, is not a valid quick method for distinguishing the two mechanisms of resistance, at least at the low levels of resistance studied.

Accumulation in the presence of VRP was up to control rates in the above five membrane mutants, but in B24 the reduced accumulation was maintained in the presence of VRP. These results are in complete agreement with the results of clonogenic assays. Therefore, although VCR accumulation alone is not a true indication of the mechanism of resistance, inclusion of VRP in the accumulation studies may give a good predictive test for the expected cross-resistance patterns, and therefore the mechanism of resistance.

We conclude that low-level VCR-resistant cells are likely to be cross-resistant to VLB, VDS, and ADR, but not resistant to CHL or 5-FU. Approximately one-half of the VCR-resistant lines are likely to show increased sensitivity to TAX, and in those lines which do not show such increased sensitivity, VCR resistance is likely to be overcome by VRP. This situation may be exploitable in chemotherapy. Finally, VCR accumulation cannot be used as a definitive guide to the type of resistance, but where there is reduced accumulation, accumulation of VCR in the presence of VRP deserves investigation since it promises to be a quick and valid method for distinguishing the mechanism of resistance and to provide a predictive test for the behavior of the tumor in vivo.

REFERENCES

1. TSURUO T. Reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics. Cancer Treat Rep 67:889–894, 1983.
2. HILL BT, and WHELAN RDH. Establishment of vincristine resistant and vindesine resistant lines of murine lymphoblasts in vitro and characterisation of their patterns of cross-resistance and drug sensitivity. Cancer Chemother Pharmacol 8:163–169, 1982.

3. BIEDLER JL, and PETERSON RHF. Altered plasma membrane glycoconjugates of Chinese hamster cells with acquired resistance to actinomycin D, daunorubicin and vincristine. *In* Molecular Actions and Targets for Cancer Chemotherapeutic Agents (Sartorelli AC, Lazo IS, and Bertino JR, eds). New York, Academic Press, 1981, chap 22, pp 453–482.
4. SKOVSGAARD T. Mechanisms of resistance to daunorubicin in Ehrlich ascites tumour cells. Cancer Res 38:1785–1791, 1978.
5. INABA M, FUJIKURA R, and SAKURAI Y. Active efflux common to vincristine and daunorubicin in vincristine resistant P388 leukaemia. Biochem Pharmacol 30:1863–1865, 1981.
6. CONTER V, and BECK WT. Acquisition of multiple drug resistance by CCRF-CEM cells selected for different degrees of resistance to vincristine. Cancer Treat Rep 68:831–839, 1984.
7. BIEDLER JL, RIEHM H, PETERSON RHF, ET AL. Membrane-mediated drug resistance and phenotypic reversion to normal growth behavior of Chinese hamster cells. J Natl Cancer Inst 55:671–680, 1975.
8. BIEDLER JL, and RIEHM H. Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic and cytogenetic studies. Cancer Res 30:1174–1184, 1970.
9. LING V, and THOMPSON LH. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. J Cell Physiol 83:103–116, 1974.
10. WILKOFF LJ, and DALMADGE EA. Resistance and cross resistance of cultured leukemia P388 cells to vincristine, adriamycin, adriamycin anologs and actinomycin D. J Natl Cancer Inst 61:1521–1524, 1978.
11. CABRAL F, ABRAHAM I, and GOTTESMAN MM. Revertants of a Chinese hamster ovary cell mutant with altered tubulin: evidence that the altered tubulin confers both colcemid resistance and temperature sensitivity on the cell. Mol Cell Physiol 2:720–729, 1982.
12. KEATES RAB, SARANGI F, and LING V. Structural and functional alterations in microtubule protein from Chinese hamster ovary cell mutants. Proc Natl Acad Sci USA 78:5638–5642, 1981.
13. LING V, AUBIN JE, CHASE A, ET AL. Mutants of Chinese hamster ovary (CHO) cells with altered colcemid binding activity. Cell 18: 423–430, 1979.
14. WARR JR, FLANAGAN DJ, and ANDERSON M. Mutants of Chinese hamster ovary cells with altered sensitivity to taxol and benzimidazole carbamates. Cell Biol Int Rep 6:455–460, 1982.
15. HOROWITZ SB, PARNESS J, SCHIFF PB, ET AL. Taxol, a new probe for studying the structure and function of microtubules. Cold Spring Harbor Symp Quant Biol 46:219–226, 1981.
16. ARMITAGE P. Analysis of variance applied to regression. *In* Statistical Methods in Medical Research. Oxford, Blackwell Scientific Publications, 1971, pp 269–301.
17. GUPTA RS. Cross-resistance of vinblastine- and taxol-resistant mutants of Chinese hamster ovary cells to other anticancer drugs. Cancer Treat Rep 69:515–521, 1985.
18. TSURUO T, KAWABATA H, NAGUNO N, ET AL. Potentiation of antitumor agents by calcium channel blockers with special reference to cross-resistance patterns. Cancer Chemother Pharmacol 15:16–19, 1985.
19. BAYSASS M, GOUVERIA J, RIBAUD P, ET AL. Phase II trial with vindesine for regression induction in patients with leukemias and hematosarcomas. Cancer Chemother Pharmacol 2:247–255, 1979.
20. MATHÉ G, MISSET JL, DE VASSAL F, ET AL. Phase II clinical trial with vindesine for remission induction in acute leukemia, blastic crisis of chronic myeloid leukemia, lymphosarcoma, and Hodgkin's disease: absence of cross-resistance with vincristine. Cancer Treat Rep 62:805–809, 1978.
21. LING V. Genetic aspects of drug resistance of somatic cells. Antibiot Chemother 23:191–199, 1978.
22. BECH-HANSEN NT, TILL JE, and LING V. Pleiotropic phenotype of colchicine resistant CHO cells. Cross resistance and collateral sensitivity. J Cell Physiol 88:23–32, 1976.
23. GIANNI L. Antitumour antibiotics, 1: anthracyclines. *In* Antitumour Drug Resistance (Fox BW, and Fox M, eds). Berlin, Springer-Verlag, 1984, pp 66–79.
24. TSURUO T, IIDA H, NAGANUMA K, ET AL. Promotion by verapamil of vincristine responsiveness in tumour cell lines inherently resistant to the drug. Cancer Res 43:808–813, 1983.
25. CORNWELL MM, SAFA AR, FELSTED RL, ET AL. Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labelling. Proc Natl Acad Sci USA 83:3847–3850, 1986.
26. SAFA AR, GLOVER CJ, MEYERS MB, ET AL. Vinblastine photoaffinity labelling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. J Biol Chem 261: 6137–6140, 1986.

Effects of Mitoxantrone and Doxorubicin on Energy Metabolism of the Rat Heart[1]

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In animal models anthracyclines and anthracenediones show similar antineoplastic activity but somewhat different cardiotoxicity. The effects of doxorubicin and the free base of mitoxantrone (NSC-279836) on the energy metabolism of the rat heart were compared. Both compounds not only reduced oxygen consumption in heart mitochondria ex vivo, but also uncoupled oxidative phosphorylation, inhibited creatine phosphate kinase, and damaged the semipermeability of the inner mitochondrial membrane (measured as creatine influx). The effects on the myocyte membrane activities, calcium transport, and Na/K, Mg, and Ca ATPases were slightly different for the two compounds. Cardiotoxicity of the two compounds may have its origin in their interference with heart cell energy metabolism. [Cancer Treat Rep 71:361-366, 1987]

The search for more potent anthracyclines with lower nonhematological toxicity was first aimed at the development of semisynthetic and synthetic derivatives of the standard agents, daunorubicin and doxorubicin. In recent years, the anthracenediones, compounds synthesized initially for quite different purposes, were discovered as a new class of anticancer drugs (1). Although structurally somewhat related to the anthracyclines, these agents differ in their ring structure and lack the daunosamine sugar group. They were found to be highly effective in various tumor models (2) and two derivatives, mitoxantrone and ametantrone, are presently under clinical evaluation.

The mechanisms of the antitumor activity of anthracyclines and anthracenediones are believed to be identical, involving intercalation into DNA and inhibition of RNA and protein synthesis (3). Thus, it is expected that toxicity affecting rapidly growing tissues, eg, bone marrow depression, will be a common feature. However, nonhematological toxicity may depend on different properties of the molecules. In multiple-dose toxicity studies in rats, mitoxantrone, used as the free-base NSC-279836, and a related anthracenedione, NSC-196473, did not cause the typical ultrastructural changes of the rat heart muscle, ie, dilatation of sarcoplasmic reticulum, myofibrillar drop-out, and dispersion of Z-line material, seen with doxorubicin (4). Similar observations were made in dogs treated with maximally tolerated doses of mitoxantrone and doxorubicin (5,6). However, in biopsy specimens of human patients treated with mitoxantrone, after a cumulative dose $\geq$ 120 mg/m^2 ultrastructural changes of the heart muscle identical to those seen with doxorubicin were noted (7).

The electron microscopic examination of the myocardium of rats treated with doxorubicin and anthracenediones also disclosed alterations of the mitochondria, mainly cristolysis, and this change was particularly marked with the latter substances (4). Cumulative toxicity in heart energy metabolism induced by anthracyclines (eg, doxorubicin, daunorubicin, and zorubicin) had been shown previously (16,17). For this reason, the effects of mitoxantrone and doxorubicin on mitochondrial energy metabolism of the rat heart were compared.

MATERIALS AND METHODS

Animals

Female rats (SIV-50, Ivanovas, Kissleg, Federal Republic of Germany) with a starting weight of 100-110 g were housed three to a cage (Macrolone No. 3) with wood shavings as bedding. The animals had free access to food (Kliba No. 343) and water. The environmental conditions were as follows: room temperature 22°C $\pm$ 1°C, relative humidity 55% $\pm$ 10%, and a 12-hour light-dark cycle. A total of 84 animals were used in these studies.

The compounds under study were doxorubicin and mitoxantrone, used as the free-base NSC-279836. The test substances were dissolved in 0.9% NaCl and given by ip injection twice a week for 4 weeks. The doses were

[1] Received Mar 5, 1986; revised Dec 8, 1986; accepted Jan 5, 1987.

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2 mg/kg for doxorubicin and 1 mg/kg for mitoxantrone. Dose selection was based on the higher overall chemotherapeutic activity of mitoxantrone in various tumor models, particularly P388 leukemia (1), and the more marked bone marrow depression induced in rats (4). Control animals received injections of equal volumes (2 ml/kg) of 0.9% NaCl. Twelve animals were randomly assigned to each group. Once a week three animals were withdrawn from the three groups at random and were killed by stunning and cervical dislocation. Hearts were excised immediately and placed into ice-cold 0.25 M sucrose/0.01 M Tris-HCl, pH 7.4, solution.

Mitochondria

Each time mitochondria were isolated from three pooled hearts by differential centrifugation following homogenization of the tissue (8). Oxygen consumption coupled to oxidative phosphorylation was measured in a Gilson Oxygraph (Gilson Medical Electronics, Middleton, WI) equipped with a Clark electrode. Succinate, β-hydroxybutyrate, 2-oxoglutarate, and DL-glutamate were used as substrates.

Creatine Phosphate Kinase and Creatine Content of Heart Mitochondria

The activity of creatine phosphate kinase (EC 2.7.3.2) was determined in heart mitochondria according to the method of Jacobus and Lehninger (9). Heart mitochondria were incubated with creatine phosphate and ADP in the presence of $MgCl_2$ at 30°C for 0-10 minutes. The reaction was stopped by adding aliquots of the incubation mixture to alpha-naphthol. The method of Eggleton et al (10) was used to determine the liberated creatine. Zero-time samples were used to determine the amount of creatine in the matrix of mitochondria.

Preparation of a Sarcolemma (SL)- and Sarcoplasmic Reticulum (SR)-Rich Fraction From the Heart

Two pooled hearts were homogenized with a Polytron in 0.1 M KCl/0.01 M Tris-HCl, pH 7.0. The membrane vesicle fraction was isolated from a 12,000 × *g* supernatant by centrifugation for 45 minutes at 31,000 × *g* and collection of the pellet (11). The fraction was shown to be at least 90% pure by electron microscopic evaluation. The method of Boelsterli et al (12) was used to measure membrane ATPases, using the buffers as described by Jones et al (11) and employing the proper inhibitors (13). The calcium-binding procedure was adapted from Jones et al (11), transporting ^{45}Ca with ATP (5 mM) as the energy source.

Protein Determination

Protein determinations on aliquots of the probes were done by the Biuret method (14) using small amounts of deoxycholate to solubilize the samples. For dilute samples the method of Lowry et al (15) was substituted.

Chemicals

All chemicals used were of reagent grade. The nucleotides were purchased from Sigma Chemical Co, St. Louis, MO. Radiolabeled compounds were purchased from Amersham International, England. Doxorubicin was a gift from Farmitalia Carlo Erba, Milan. NSC-279836, the free base of mitoxantrone, was provided by the National Cancer Institute, Bethesda, MD.

RESULTS

Each week animals were withdrawn at random. Mitochondria were isolated from the heart and assayed for electron transfer activities (oxygen consumption) and oxidative phosphorylation. The results of these experiments are summarized in figure 1. The data given are those for β-hydroxybutyrate, but they are representative for all four substrates tested (succinate, β-hydroxybutyrate, 2-oxoglutarate, and DL-glutamate). With both drugs the mitochondria of the heart showed a gradual reduction in electron transfer activities (state III) as well as the respiratory control index according to Chance (25) and a concomitant uncoupling of oxidative phosphorylation as indicated by a decrease of the ADP/O ratio. Doxorubicin and mitoxantrone appeared to have similar effects on the mitochondria (tested ex vivo);

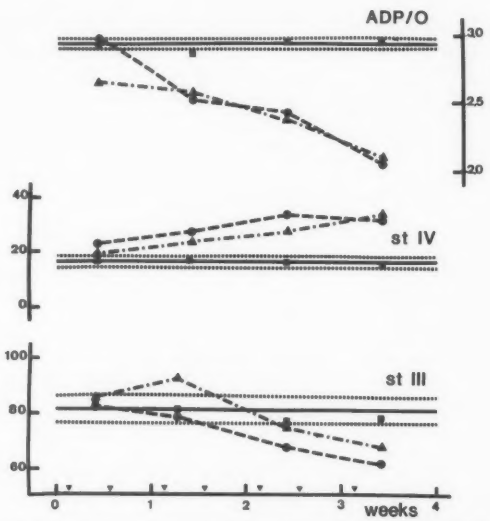

FIGURE 1.—Effect of mitoxantrone and doxorubicin on heart mitochondrial metabolism in rats. ADP/O = measure of oxidative phosphorylation capacity of mitochondria (with β-hydroxybutyric acid as substrate, the maximal capacity is 3 ATP formed/L of oxygen consumed); st III and st IV = respiration of mitochondria in state III and state IV according to Chance (25), expressed in nmols of oxygen consumed/mg of protein/min; ▼ = time of treatment of rats; ▲–·–·–▲ = doxorubicin dose, 2 mg/kg ip; ●——● = mitoxantrone dose, 1 mg/kg ip; ■ = activities measured with control animals; and ═══ = activities of mitochondria from pooled control animals, treated with 2 ml/kg of 0.9% NaCl ip, mean ± 2 SD.

TABLE 1.—Effect of doxorubicin and mitoxantrone on the creatine phosphate kinase of heart mitochondria*

Week	No. of — Doses	No. of — Days	Controls (2 ml/kg of 0.9% NaCl ip)	Doxorubicin (2 mg/kg ip)	Mitoxantrone (1 mg/kg ip)
1	1	+2	8.37 ± 0.12	7.33 ± 0.45	6.92 ± 0.54
2	3	+2	8.42 ± 0.09	6.25 ± 0.52	6.50 ± 0.50
3	5	+2	8.32 ± 0.07	6.58 ± 0.75	6.50 ± 0.50
4	7	+2	8.40 ± 0.05	6.75 ± 0.39	6.73 ± 0.34

*$\times 10^{-4}$ mol/min/mg of protein. Values = mean ± 1 SD (10 determinations).

however, it must be kept in mind that mitoxantrone was administered at half the dose of doxorubicin (4).

Concomitantly, creatine phosphate kinase activity of heart mitochondria was inhibited (table 1). Both drugs caused an immediate drop of the activity already after the first dose, followed by only a slight further decrease in activity during the remaining weeks of treatment. The creatine content of heart mitochondria (table 2), an index of loss of membrane semipermeability, increased steadily to reach about 130% of controls after 4 weeks of treatment with both drugs.

In a further set of two separate experiments, a myocyte membrane fraction (SL + SR) was isolated from heart tissue and assayed for calcium transport activities (measured as ^{45}Ca binding to membrane vesicles) and for various membrane ATPases (Na/K, Mg, and Ca). The results are summarized in tables 3 and 4. Doxorubicin (table 3) caused an immediate inhibition of Ca transport activities of 10% after only one dose, which increased gradually to 25% after 4 weeks. With mitoxantrone, Ca transport activities were inhibited in the first week by 17% and stimulated by 25% in the second and 28% in the third week. They returned close to control values in the fourth week of drug treatment.

The data concerning ATPase activities of these membrane fractions are summarized in table 4. Doxorubicin initially inhibited all membrane ATPases (Na/K, Mg, and Ca), 66% of controls for Na/K, 69% for Mg, and 84% for Ca ATPase. The various ATPases returned gradually to control values in the following weeks despite continued drug treatment. Mitoxantrone caused an immediate drop in activity of Na/K ATPase of about 59% compared to controls, and this change was maintained over the 4-week treatment period. The initial decrease of Mg ATPase and Ca ATPase was less marked (12% and 13%, respectively), but the activities dropped progressively to lower levels during the remaining treatment period.

TABLE 2.—Effect of doxorubicin and mitoxantrone on the creatine content of heart mitochondria*

Week	No. of — Doses	No. of — Days	Controls† (2 ml/kg of 0.9% NaCl ip)	Doxorubicin‡ (2 mg/kg ip)	Mitoxantrone‡ (1 mg/kg ip)
1	1	+2	8.20 ± 0.18	8.77	8.94
2	3	+2	8.15 ± 0.20	10.00	9.51
3	5	+2	8.21 ± 0.17	9.75	10.82
4	7	+2	8.25 ± 0.21	10.61	10.99

*$\times 10^{-6}$ mol/mg of protein. Values = mean ± 1 SD.
†4 determinations.
‡Average of 2 determinations.

TABLE 3.—Effects of doxorubicin and mitoxantrone on calcium transport activities of heart myocyte membrane vesicles (SL + SR)*

Week	No. of — Doses	No. of — Days	Doxorubicin Controls (2 ml/kg of 0.9% NaCl ip)	Doxorubicin Medicated (2 mg/kg ip)	Mitoxantrone Controls (2 ml/kg of 0.9% NaCl ip)	Mitoxantrone Medicated (1 mg/kg ip)
1	1	+2	6.95 ± 0.21	6.25 ± 0.45	9.08 ± 0.51	7.52 ± 0.59
2	3	+2	7.05 ± 0.31	5.83 ± 0.26	8.89 ± 0.32	11.14 ± 0.69
3	5	+2	6.88 ± 0.41	5.79 ± 0.31	9.12 ± 0.26	11.69 ± 0.65
4	7	+2	7.15 ± 0.50	5.35 ± 0.41	9.01 ± 0.40	9.93 ± 0.83

*μmols of ^{45}Ca bound/min/mg of protein. Values = mean ± 1 SD (10 determinations).

TABLE 4.—Effects of doxorubicin and mitoxantrone on ATPases (

			Doxorubicin				
	No. of —		Controls (2 ml/kg of 0.9% NaCl ip)			Medicate (2 mg/kg	
Week	Doses	Days	Na/K	Mg	Ca	Na/K	Mg
1	1	+2	99 ± 2	629 ± 6	621 ± 7	65 ± 2	432 ± 11
2	3	+2	95 ± 3	625 ± 2	612 ± 9	57 ± 4	512 ± 14
3	5	+2	96 ± 5	618 ± 10	615 ± 2	89 ± 7	529 ± 11
4	7	+2	100 ± 2	621 ± 8	620 ± 8	105 ± 5	613 ± 13

*nmols of $^{32}PO_4^{-2}$ released/min/mg of protein. Values = mean ± 1 SD (4 determinations).

Pases (Na/K, Mg, and Ca) of heart myocyte membrane vesicles (SL + SR)*

		Mitoxantrone					
edicated ng/kg ip)		Controls (2 ml/kg of 0.9% NaCl ip)			Medicated (1 mg/kg ip)		
Mg	Ca	Na/K	Mg	Ca	Na/K	Mg	Ca
2 ± 11	523 ± 12	94 ± 3	645 ± 31	717 ± 40	39 ± 3	567 ± 11	623 ± 19
2 ± 14	592 ± 12	90 ± 5	638 ± 22	709 ± 35	43 ± 4	496 ± 15	523 ± 21
9 ± 11	605 ± 25	96 ± 4	641 ± 34	712 ± 41	65 ± 3	451 ± 12	458 ± 22
3 ± 13	684 ± 15	89 ± 3	635 ± 12	708 ± 25	48 ± 2	496 ± 13	509 ± 18

s).

The animals treated with doxorubicin or mitoxantrone were in reasonably good health at the end of the 4-week treatment period (slight weight loss, shaggy fur, and very little ascites). Treatment with mitoxantrone caused a blue discoloration of the skin and all tissues; even the mitochondria isolated from heart appeared blue.

DISCUSSION

In previous experiments with daily dosing doxorubicin had caused a reduction in electron transfer activities in rat heart mitochondria and no uncoupling of oxidative phosphorylation (16,17). Using a better-tolerated regimen with two weekly doses of 2 mg/kg but the same cumulative dose, reduction in electron transfer activities and uncoupling of oxidative phosphorylation occurred. The degree of uncoupling observed in heart mitochondria was the same as that measured in kidney mitochondria. Mitochondrial creatine phosphate kinase, one of the enzymes transferring high-energy phosphates from ATP generated internally in the mitochondrial matrix to form creatine phosphate outside the mitochondria, was also impaired by doxorubicin. It is probable that this effect would reduce the energy available for muscle contraction even further. The increase in mitochondrial creatine content indicates a loss of mitochondrial membrane semipermeability. This could also be interpreted as a consequence of a diminished production of mitochondrial high-energy phosphates.

The dose of mitoxantrone selected for this study was 50% that of doxorubicin. This was not only dictated by higher bone marrow toxicity (4), but also by a more potent chemotherapeutic activity in various tumor models (1,2). With this dose, mitoxantrone affected electron transfer activities slightly less than and oxidative phosphorylation and creatine phosphate kinase activity to the same extent as doxorubicin. The creatine content of heart mitochondria, which is a sensitive indicator of membrane integrity, increased steadily with both treatments.

Calcium binding to SL and SR membrane vesicles was consistently, although moderately, inhibited by doxorubicin, whereas mitoxantrone caused only an initial inhibition which was followed by transient stimulation and a return to normal levels. The differences between the control groups of the two experiments that were performed at different times are not explained. Some differences were also observed in regard to the effects on various ATPases: Na/K ATPase was markedly inhibited and Mg and Ca ATPases were moderately inhibited by mitoxantrone, and the effect was present during the whole experiment. With doxorubicin, the inhibitory effects were moderate and disappeared towards the end of treatment.

From these observations it is concluded that the two antitumor drugs caused similar disturbances of enzymes and membrane functions involved in myocardial energy metabolism. The major differences were observed with regard to calcium binding to SL and SR membrane vesicles, which was much less affected in rats treated with mitoxantrone, and the activities of ATPases, which were more consistently inhibited by the anthracenedione derivative. These findings are in agreement with those of Neri et al (18), who compared the effects of doxorubicin and mitoxantrone on rat heart slices in vitro. Both drugs caused progressive reduction in oxygen uptake coupled to a loss of ATP and CrP from the tissue.

In the literature, various anthracenediones (NSC-279836, mitoxantrone, and ametantrone) have been compared repeatedly with compounds of the anthracycline group. The principal goal was the elucidation of the mechanism of action and a comparison of their adverse effects, among which cardiotoxicity is of utmost importance for both groups. Various tumors showed cross resistance to the two groups of cytostatics, in spite of the differences now known in their mechanisms of action. Interactions with double-stranded DNA were studied by Kapuscinski and Darzynkiewicz (19) and Lown et al (3), genotoxicity by Au et al (20), and the interaction with microsomal NADPH-cytochrome P_{450} reductase by Kharasch and Novak (21). The latter investigators studied the metabolic activation of free-radical semiquinone intermediates with subsequent formation of superoxide. They noted that the anthracenediones had a behavior opposite to the one known for anthracyclines; ie, they stimulate NADPH oxidation and superoxide generation only very slightly, but interact with the catalytic site of cytochrome P_{450} reductase. Mimnaugh et al (22) and Doroshow et al (23) showed that doxorubicin and mitoxantrone had the opposite effect on lipid peroxidation in microsomes isolated from control hearts as well as from vitamin E-deficient rats. Doxorubicin was shown to be a potent stimulator of lipid peroxidation, whereas mitoxantrone almost completely suppressed lipid peroxidation in normal as well as in α-tocopherol-depleted heart tissue. Mitoxantrone and ametantrone were even shown to counteract the stimulation of lipid peroxidation by doxorubicin of rabbit liver microsomes in vitro (24). NADPH oxidation was not affected by NSC-279836 but was greatly stimulated by doxorubicin. Neither of the two compounds had any effect on the NADPH-cytochrome P_{450} reductase activity.

From these studies it is apparent that doxorubicin and mitoxantrone have similar effects on mitochondrial energy metabolism in the rat, and this can be related to the ultrastructural damage of mitochondria observed in the same species with both drugs (4). On the other hand, the two compounds differ greatly with regard to their stimulant properties on lipid peroxidation. Since dilatation and vacuole formation of the SR is a typical feature of doxorubicin toxicity in all animal species, but was not or only marginally present in mitoxantrone-treated rats (4) and dogs (6), it can be concluded that the permeability changes that facilitate the influx of fluid into the SR may be due to peroxidative membrane damage. Which of the two biological mechanisms of experimental myocardial lesions, impairment of mitochondrial function or membrane peroxidation, is of greater importance for

the development of cardiotoxicity in man can only be determined when more information on the hazards of anthracenediones to induce cardiomyopathy in man is available.

REFERENCES

1. CHENG CC, ZBINDEN G, and ZEE-CHENG RKY. Comparison of antineoplastic activity of aminoethylaminoanthraquinones and anthracycline antibiotics. J Pharm Sci 68:393-396, 1979.
2. YOUNG CW, and RAYMOND V. Clinical assessment of the structure-activity relationship of anthracyclines and related synthetic derivatives. Cancer Treat Rep 70:51-63, 1986.
3. LOWN JW, MORGAN AR, YEN SHF, ET AL. Characteristics of the binding of the anticancer agents mitoxantrone and ametantrone and related structures to deoxyribonucleic acids. Biochemistry 24: 4028-4035, 1985.
4. ZBINDEN G, and BEILSTEIN AK. Comparison of cardiotoxicity of two anthracenediones and doxorubicin in rats. Toxicol Lett 11:289-297, 1982.
5. HENDERSON BM, DOUGHERTY WJ, JAMES VC, ET AL. Safety assessment of a new anticancer compound, mitoxantrone, in beagle dogs: comparison with doxorubicin. I. Clinical observations. Cancer Treat Rep 66:1139-1143, 1982.
6. SPARANO BM, GORDON G, HALL C, ET AL. Safety assessment of a new anticancer compound, mitoxantrone, in beagle dogs: comparison with doxorubicin. II. Histologic and ultrastructural pathology. Cancer Treat Rep 66:1145-1158, 1982.
7. UNVERFERTH DV, UNVERFERTH BJ, BALCERZAK SP, ET AL. Cardiac evaluation of mitoxantrone. Cancer Treat Rep 67:343-350, 1983.
8. BACHMANN E, WEBER E, POST M, ET AL. Biochemical effects of gum arabic, gum tragacanth, methylcellulose and carboxymethylcellulose-Na in rat heart and liver. Pharmacology 17:39-49, 1978.
9. JACOBUS WE, and LEHNINGER AL. Creatine kinase of rat heart mitochondria. J Biol Chem 248:4803-4810, 1973.
10. EGGLETON P, ELSDEN SR, and GOUGH N. The estimation of creatine and diacetyl. Biochem J 37:526-529, 1943.
11. JONES LR, BESCH HR, and WATANABE AM. Monovalent cation stimulation of Ca^{+2} uptake by cardiac membrane vesicles. J Biol Chem 252:3315-3323, 1977.
12. BOELSTERLI UA, RAKHIT G, and BALAZS T. Modulation by *S*-adenosyl-L-methionine of hepatic Na^+, K^+-ATPase, membrane fluidity, and bile flow in rats with ethinyl-estradiol-induced cholestasis. Hepatology 3:12-17, 1983.
13. BACHMANN E, WEBER E, and ZBINDEN G. Biochemical mechanism of quinidine cardiotoxicity. Cardiovasc Pharmacol 8:826-831, 1986.
14. GORNALL AG, BARDAWILL CT, and DAVID MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751-766, 1949.
15. LOWRY OH, ROSEBROUGH NJ, FARR AL, ET AL. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951.
16. BACHMANN E, WEBER E, and ZBINDEN G. Effects of seven anthracycline antibiotics on electrocardiogram and mitochondrial function of rat heart. Agents Actions 5:383-393, 1975.
17. BACHMANN E, and ZBINDEN G. Effect of doxorubicin and rubidazone on respiratory function and Ca^{2+}-transport in rat heart mitochondria. Toxicol Lett 3:29-34, 1979.
18. NERI B, CINI-NERI G, and D'ALTERION M. Effect of anthracyclines and mitoxantrone on oxygen uptake and ATP intracellular concentration in rat heart slices. Biochem Biophys Res Commun 125:954-960, 1984.
19. KAPUSCINSKI J, and DARZYNKIEWICZ Z. Interactions of antitumor agents ametantrone and mitoxantrone (Novatrone) with double-stranded DNA. Biochem Pharmacol 34:4203-4213, 1985.
20. AU WW, BUTLER MA, MATNEY TS, ET AL. Comparative structure-genotoxicity study of three aminoanthraquinone drugs and doxorubicin. Cancer Res 41:376-379, 1981.
21. KHARASCH ED, and NOVAK RF. Anthracenedione activation by NADPH-cytochrome P_{450} reductase: comparison with anthracyclines. Biochem Pharmacol 30:2881-2884, 1981.
22. MIMNAUGH EG, TRUSH MA, GINSBERG E, ET AL. Differential effects of anthracycline drugs on rat heart and liver microsomal reduced nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation. Cancer Res 42:3574-3582, 1982.
23. DOROSHOW JH, and DAVIES KJA. Comparative cardiac metabolism by anthracycline antibiotics, mitoxantrone, bisantrene, 4'-(9-acridinylamino)-methanesulfon-*m*-anisidide, and neocarzinostatin. Biochem Pharmacol 32:2935-2939, 1983.
24. KHARASCH ED, and NOVAK RF. Inhibition of Adriamycin-stimulated microsomal lipid peroxidation by mitoxantrone and ametantrone, two new anthracenedione antineoplastic agents. Biochem Biophys Res Commun 108:1346-1352, 1982.
25. CHANCE B, and WILLIAMS GR. Respiratory enzymes in oxidative phosphorylation. J Biol Chem 217:395-407, 1955.

Increased Therapeutic Efficacy and Reduced Toxicity of Doxorubicin Linked to Pyran Copolymer via the Side Chain of the Drug[1,2]

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Doxorubicin was covalently linked to divinyl ether-maleic anhydride copolymer (pyran copolymer) in its polycarboxylate form via the methylketone side chain through a nucleophilic substitution reaction of the 14-bromo derivative of the drug. The drug conjugated to the synthetic polyanionic polymer was tested for antitumor activity in a range of experimental murine tumor systems. When administered ip to mice bearing ip implanted tumors (P388 leukemia or macrophage tumor J774), the polymer-linked drug was superior to free doxorubicin and daunorubicin in increasing the life span of treated animals. Treatment with the conjugate also resulted in an improvement in survival time of mice bearing ascitic M50 tumor, although the effects of a single dose of free drug, in the range of maximum tolerated doses, were marginal. When given iv, the conjugate was more effective than free drug against systemic Gross leukemia. The therapeutic advantage of the polymer-linked doxorubicin over free drug was more marked when a multiple treatment schedule was used. Studies in vitro showed that the drug following covalent fixation to the polymer had only marginally decreased cytotoxicity against HeLa and P388 cells when compared with that of free anthracycline. This effect paralleled the lack of reduction in in vivo potency. Moreover, the covalent linkage of the drug to synthetic polymer reduced drug toxicity. This effect was more marked with the ip route of administration than with the iv route. [Cancer Treat Rep 71:367-373, 1987]

The potential use of macromolecules as carriers of antitumor drugs has been examined by several investigators in an attempt to produce a preferential deposition of the drug-carrier conjugate in the site of tumor cells and to provide sustained plasma levels of drug because of its slow release from the polymeric carrier (1,2). Although the ultimate goal of this approach remains the development of a drug delivery system characterized by highly selective drug action, restriction of drug distribution and/or action only to the neoplastic target tissue is still an unresolved problem. Linkage of cytotoxic drugs to suitable macromolecular carriers, although lacking the features of specificity, has been found to provide therapeutic advantages over free drugs (3). Synthetic polymers have been proposed as carriers of antitumor agents since they offer many possibilities in macromolecular drug design and structural modifications. They have also been used for drug targeting by fixation to more selective transport units (ie, antibodies), thereby allowing delivery of a considerably large number of drug molecules without loss of biological activity of the transport system (4,5). In particular, polyanionic polymers, especially polycarboxylic acid polymers, have often been used as drug carriers (6).

We have recently reported that the covalent linkage of doxorubicin (DX) to poly-L-aspartic acid resulted in reduction of anthracycline toxicity, whereas the drug maintained or improved the antitumor efficacy (7-9). Several polyanionic polymers are known to exhibit a wide range of biological activity (10), including immunostimulating and antitumor activity (11). Among polyanionic polymers, the synthetic divinyl ether-maleic anhydride copolymer [pyran copolymer (Py)] has been extensively studied (12,13). In addition, this polymer has been used as a drug carrier in the attempt to exploit possible drug-polymer synergism (14,15).

In the present study we examined the antitumor activity of the DX-pyran copolymer conjugate (DX-Py) in

[1] Received July 14, 1986; revised Dec 9, 1986; accepted Jan 5, 1987.

[2] Supported in part by a grant (Progetto Finalizzato Oncologia) from the Consiglio Nazionale delle Ricerche, Rome.

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[4] We thank Giuseppina Savi and Stella Tinelli for technical assistance and Mrs. Grazia Barp and Ms. Betty Johnston for editorial assistance and manuscript preparation.

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a number of experimental tumor models. The drug was covalently linked to hydrolyzed polymer using a procedure similar to that described for drug-anionic polyamino acid conjugation (8).

MATERIALS AND METHODS

Drugs

DX, daunorubicin (DR), and 14-bromo-DR were supplied by Farmitalia Carlo Erba (Milan, Italy). The Py used in the synthesis of the polymeric derivative of DX was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) and had an average molecular weight of 3.6×10^4.

Preparation of DX-Py

Py (100 mg suspended in 5 ml of distilled water) was completely dissolved and converted to the polycarboxylate form by hydrolysis with 0.2 N aqueous NaOH solution (pH > 12) for at least 15 minutes. Complete hydrolysis of the maleic anhydride was confirmed by potentiometric titration. After hydrolysis, the pH of the solution was adjusted to 8.5. 14-Bromo-DR (20–50 mg freshly dissolved in a small volume of methanol) was added to the polymer solution under stirring. The reaction was carried out at room temperature. The acid produced during the nucleophilic substitution reaction was neutralized by the addition of a dilute aqueous solution of NaOH. The reaction mixture was stirred for 2 hours and then adjusted to pH 7.0. 14-Bromo-DR reacts with carboxyl groups of the polymer to yield a doxorubicinyl ester through the side chain. 14-Bromo-DR and any free antibiotic were removed by ion-exchange chromatography on Dowex 50W-X2 columns. The DX conjugate was diluted with water to reduce the polymer concentration. Thin-layer chromatography (using chloroform:methanol:acetic acid, 80:20:4 by volume for elution) and high-pressure liquid chromatography on a C-18 reverse-phase column (using water:acetonitrile, 66:34 by volume, adjusted to pH 3.8 with phosphoric acid, as mobile phase at a flow rate of 1.5 ml/min; fluorimetric detection: excitation, 500 nm; and emission, 590 nm) were used to detect free anthracycline derivatives in the samples of the polymeric derivative. Samples showing residual free drug were chromatographed again on Dowex 50W-X2 columns. The concentration of bound drug in the polymeric derivative was estimated by absorbance at 480 nm using an extinction coefficient, $E^{1\%}_{1cm} = 173$. The polymer content in the conjugates was determined by dry weight after exhaustive dialysis of the conjugate against distilled water to remove any salt. The titration data were also used to calculate the available carboxyl groups and therefore the extent of drug substitution. These methods gave identical results. The polymeric derivative used for in vivo experiments usually contained ≤ 40% DX by weight (range of drug-to-polymer molar ratio, 15:30).

Since the anhydride function could also react with the drug amino group to produce an amide linkage, this type of reaction has been excluded on the basis of the following observations. (*a*) Titration curves indicated complete hydrolysis of the anhydride under the coupling reaction conditions. (*b*) The presence of residual anhydride was also excluded since there was no formation of the N-acyl derivative when free aminoglycosides were mixed and incubated with alkali-hydrolyzed polymer under standard reaction conditions; indeed, the mixture was easily separated by physical procedures (solvent extraction and/or ion-exchange chromatography).

Cell Survival Determination

HeLa cells were exposed to the drug for 24 hours. Survival was determined by the ability of treated cells to form colonies. After treatment, the medium was removed and cells were washed and suspended in Eagle's minimal essential medium containing 10% fetal calf serum, and plated colonies were counted after 8 days of incubation.

After drug treatment, P388 leukemia cells were centrifuged, resuspended, and cultured in drug-free medium for 72 hours and then counted with a Coulter counter.

Animals

The male and female mice that were employed throughout the experiments were obtained from Charles River Laboratories (Calco, Como, Italy), weighed between 17 and 22 g, and were maintained under conventional conditions.

Tumors

The macrophage tumor J774 was serially maintained in ascitic form in female BALB/c mice. For chemotherapy experiments, 10^6 cells/mouse were injected ip in female BALB/c or CDF_1 (BALB/c × DBA/2) mice (16).

The murine reticulum cell sarcoma (17) M5076/73a (M5) was maintained in the ascitic form by implantation of 2×10^6 cells/mouse ip in female C57BL mice. Experiments were carried out on BDF_1 (C57BL × DBA/2) mice inoculated with the same number of cells.

The P388 leukemia experiments were carried out in BDF_1 mice inoculated ip with 1×10^6 cells/mouse, whereas the tumor line was maintained in DBA/2 mice.

The experiments with Gross leukemia were carried out in C3H/He mice inoculated iv with 2×10^6 cells/mouse. The same strain was used for serial tumor passages.

Treatment

For antitumor testing, DR and DX were dissolved in distilled water to a concentration that allowed the dose to be given in a volume of 0.1 ml/10 g of body weight and administered iv or ip as indicated. Drug solutions were freshly prepared before use. Polymeric derivatives were dissolved in distilled water or physiologic saline

for ip or iv administration, respectively. Mice were treated with a single dose of drug 24 hours after tumor cell inoculation.

Evaluation of Antitumor Activity

For antitumor activity experiments, eight to ten mice per group were used. The effect on survival was expressed as percent ILS (increase in life span) calculated as follows:

$$\text{ILS} = [(T/C) - 1] \times 100,$$

where T/C is the median survival time (MST) of dying mice only in the treated group (T) divided by the MST of the untreated control group (C). Long-term (60–90 days) survivors (LTS) were considered cured and were noted separately. Deaths that occurred in treated animals before the first death of control untreated animals and deaths with no signs of tumor and reduced spleen and liver size were attributed to drug toxicity.

Statistical Analysis

Student's t test was used for statistical comparisons.

RESULTS

Figure 1 compares the cytotoxic effects of free and polymer-linked DX on P388 and HeLa cells after exposure to the drugs for 24 hours. For both cell lines, the cytotoxic potency of the anthracycline was somewhat reduced following conjugation. This was expected, since decreased cytotoxicity for the conjugate form has been reported for several drugs, including DX (9,15,18). However, the observed reduction was marginal and less marked than observed for the DX-poly-L-aspartic acid conjugate (19).

The activity of DX-Py against ip (P388 leukemia, J744 lymphoma, and M5 reticulum cell sarcoma) and iv (Gross leukemia) implanted tumor models following a single administration was compared with that of free DX and DR. DR was included as a reference compound of this class. Drugs were administered by the same route used for tumor cell inoculation.

Against ip P388 leukemia, the polymeric derivative of DX was tested at various doses (expressed as DX content in the conjugate) and showed dose-dependent antitumor activity. The lowest dose tested (8 mg/kg) maintained antitumor effect (% ILS = 150 compared to % ILS = 180 produced by 10 mg/kg of DX), thus indicating no appreciable loss of drug potency caused by covalent linking to the polymer. However, an appreciable proportion of tumor-free survivors was observed only at higher doses of the conjugate. The low toxicity of the ip administered DX-Py allowed a marked increase in drug dose. High doses (around 40 mg/kg) were well-tolerated (no toxic deaths), thus indicating a fourfold reduction in drug toxicity following conjugation. The effectiveness of the polymeric derivative is best illustrated by survival curves of typical experiments that also compare the

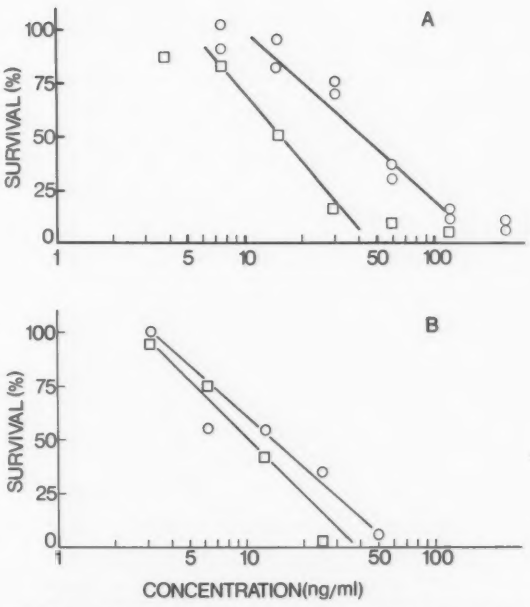

FIGURE 1.—Effects of free (□) and polymer-linked (○) DX on survival of P388 (A) and HeLa (B) cells. Cells were exposed to drug for 24 hrs.

results obtained with the optimum dose of free drugs (fig 2). Although the treatment with DX-Py was characterized by a wide range of effective doses, the best therapeutic effects were obtained at 35–40 mg/kg. The highest doses tested (48 and 60 mg/kg) were not well-tolerated (six of 19 toxic deaths for both). DX-Py was markedly more active than both free DX and DR and produced large increases in ILS (about 300%, in the range of optimal doses) and cure rates (LTS, Day 60) as high as 40%. Free DX and DR only occasionally produced LTS in this tumor system. The polymer itself had only marginal activity against P388 leukemia (maximum % ILS = 65 at 80–160 mg/kg). Although a detailed study of dose-response for combination was not carried out, simultaneous administration of free drug and polymer produced antitumor effects comparable to those of free drug alone (data not shown). In addition, Py only marginally influenced the toxicity of the free anthracycline antibiotic.

Again, when examined against the ip J744 tumor, DX-Py showed high levels of antitumor activity (fig 3). Although DX in this model produced an appreciable proportion (17%) of LTS (at Day 90), the increase in survival time observed with DX-Py remained higher than that obtained with free DX at their respective optimal doses (30–38 vs 6.6 mg/kg). DR showed only moderate activity in this system (the best % ILS value obtained in the two separate experiments was 40).

With ascitic M5 tumor (table 1), polymer-bound DX exerted a somewhat greater therapeutic effect than free drug. However, the effects of a single dose of free

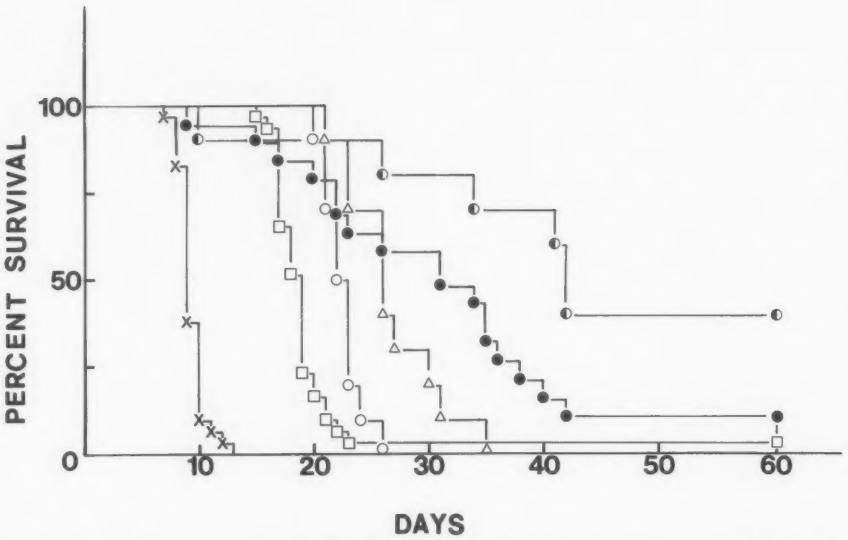

FIGURE 2.—Survival curves of mice with P388 leukemia that received single treatment ip on Day 1 after tumor transplantation: □ = 4 mg/kg of DR (29 mice); △= 10 mg/kg of DX (10 mice); ○ = 8 mg/kg of DX-Py (10 mice); ◐ = 38 mg/kg of DX-Py (10 mice); ● = 48 mg/kg of DX-Py (19 mice); and x = untreated controls (29 mice). In the case of the conjugate, dose is expressed as dose of DX content. For DX and DR, included for comparison, data obtained at the optimal dose for each drug are presented.

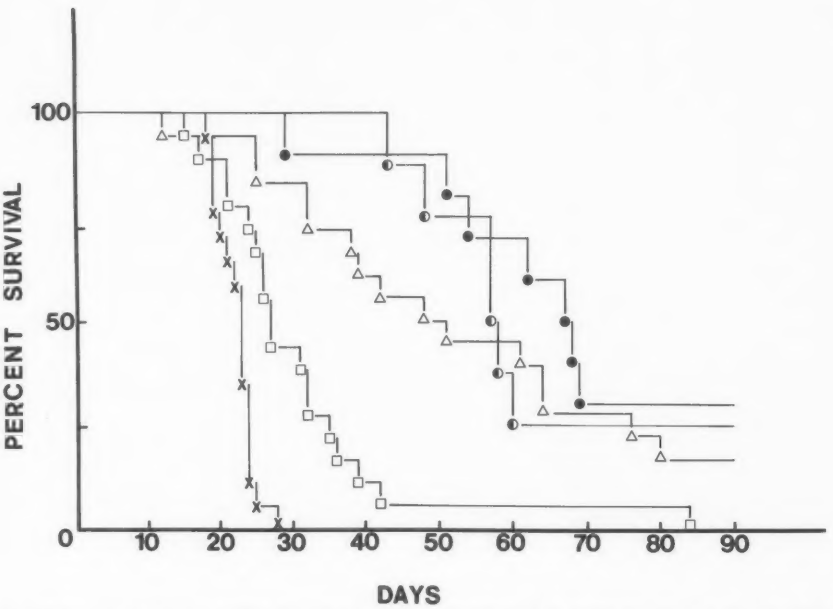

FIGURE 3.—Survival curves of mice bearing the J774 tumor that received single treatment ip on Day 1 after tumor transplantation: □ = 3.3 mg/kg of DR (18 mice); △ = 6.6 mg/kg of DX (18 mice); ◐= 30 mg/kg of DX-Py (8 mice); ● = 38 mg/kg of DX-Py (10 mice); and x = untreated controls (17 mice). In the case of the conjugate, dose is expressed as dose of linked DX. For DX and DR, included for comparison, data obtained at the optimal dose for each drug are presented.

TABLE 1.—Effect of free and polymer-linked DX on M5 tumor*

Drug	Dose (mg/kg)†	% ILS‡	*P* value§	Toxic deaths (*n* = 10)
DX	6.6	43		—
	10.0	48		1
DX–Py	6.6	48		—
	10.0	60		—
	20.0	64		—
	40.0	85	< 0.01	—
DX+Py	6.6 + 26.4	52		—
	10.0 + 40.0	69	< 0.05	—
Py	100.0	65		—

*Mice were treated ip on Day 1 after tumor transplantation (2×10^6 cells/mouse ip). Each group consisted of 10 mice.

†In conjugate and mixture, dose is expressed as DX content; polymer-to-drug ratio (wt/wt) was 4.

‡MST of control mice was 23 days.

§As compared to optimal dose of free DX, using Student's t test.

DX, in the range of the maximum tolerated doses (6–10 mg/kg), were marginal, as previously reported (7). The increase in survival time of animals treated with a mixture of DX and polymer carrier (at the weight ratio present in the conjugate) was higher than that produced by DX alone. The drug-free polymer also produced an appreciable antitumor effect (% ILS = 65) at 100 mg/kg. The therapeutic advantage of the polymer-linked drug is probably related to the large amount of drug administered. However, the advantage appeared marginal, probably as a consequence of the relative resistance of this tumor to therapy with DX.

The effects of iv treatment with DR, DX, and DX-Py on Gross leukemia are shown in table 2. Under our experimental conditions with a single-treatment schedule, the maximum effect induced by DX-Py was an ILS value of 183% at the optimal dose of 17.3 mg/kg. The same dose of free DX resulted in a similar increase (158%) in the survival time together with three of ten toxic deaths. The difference between the effects of the optimal doses (17.3 mg/kg of DX-Py and 14.4 mg/kg of DX) was statistically significant ($P < 0.05$). In addition, LTS were observed occasionally in animals treated with DX-Py. The polymer itself exerted only a marginal effect against this systemic leukemia (% ILS = 33 at the maximum tolerated single dose for unconjugated polymer, ie, around 40 mg/kg iv). However, the ratio between the highest nontoxic doses of polymer-linked and free drug after iv treatment was lower than that observed following ip injection, thus indicating a marginal reduction in drug toxicity in the polymeric form using the iv route of drug administration. Relevant to this point is the observation that, when mortality was recorded after 24 hours, apparently the acute lethal toxicity of the carrier polymer was also reduced following covalent fixation of the anthracycline, since iv doses of drug-linked polymer > 40 mg/kg were tolerated (DL50 of the polymer in the conjugate form was around 130 mg/kg, data not shown). Multiple treatments of Gross leukemia with the conjugate resulted in a more marked improvement in efficacy (ie, increase in survival time) compared with free drug (fig 4).

DISCUSSION

In most of the murine tumor systems tested in this work, pyran copolymer-linked DX exhibited therapeutic effects superior to those produced by free drug using the same treatment schedule. The covalent linkage of the drug to polymer also decreased the anthracycline toxicity, as already reported for another anionic polymer using the same method of conjugation (8,9). However, unlike the DX-poly-L-aspartic acid conjugate, this effect was more marked following ip than iv administration. It is possible that the different systemic toxicity of the polymeric derivative DX-Py using different routes of administration is the result of an enhanced toxicity of the polymer itself after iv administration (11,19). Reduction in toxicity of antitumor agents following conjugation with Py was not a general finding, since polymeric derivatives of cyclophosphamide (20) and of methotrexate (21) have been found to be more toxic than corresponding free monomeric derivatives. Free DX in its range of optimal doses (8–15 mg/kg) and polymer-linked DX at similar doses had comparable activity against ip P388 leukemia, thus indicating no appreciable reduction in drug potency following conjugation. This finding is consistent with a marginal reduction in cytotoxic activity on P388 and HeLa cells. Since in other studies the covalent attachment of low-molecular-weight

TABLE 2.—Comparison of the effects of DR, DX, and its polymeric derivative after single iv treatment on Gross leukemia*

Drug	Dose (mg/kg)†	% ILS‡	Toxic deaths (*n* = 10)
DR	14.4	66	—
	17.3	83§	—
	20.7	91	1
	24.8	116	1
DX	10.0	91	—
	12.0	116	—
	14.4	100	—
	17.3	158	3
DX-Py	14.4	83§	—
	17.3	183¶	—
	20.7	150§	2
	24.8	100	3
	29.7	91	7
Py	40.0	33	0

*C3H/He mice received 2×10^6 cells iv on Day 0. Each group consisted of 10 mice.

†In the conjugate, dose is expressed in DX equivalents. In this preparation, the drug-to-polymer molar ratio was 27, corresponding to a polymer-to-drug ratio (wt/wt) of 2.3.

‡MST of control mice was 6 days.

§LTS in 1 of 10 mice = 60 days.

¶$P < 0.01$, as compared to the optimal dose of free DX (14.4 mg/kg).

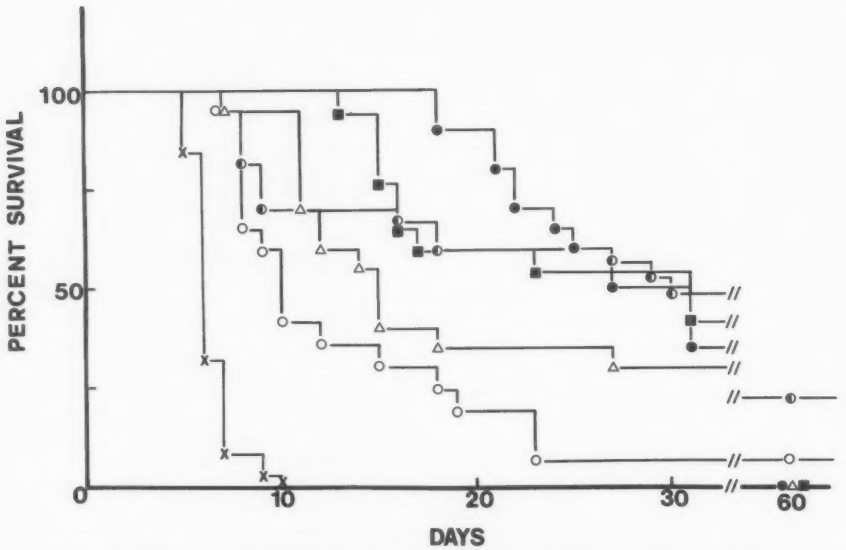

FIGURE 4.—Effect of multiple treatments with free and polymer-bound DX on survival time of mice bearing systemic Gross leukemia. Mice were treated iv on Days 1, 4, and 7 after tumor transplantation (2×10^6 cells iv on Day 0): △ = 5 mg/kg of DX (20 mice); ○ = 8 mg/kg of DX-Py (17 mice); ◐ = 12 mg/kg of DX-Py (27 mice); ● = 16 mg/kg of DX-Py (20 mice); ■ = 18 mg/kg of DX-Py (17 mice); and x = untreated controls (37 mice). In the case of the conjugate, the dose is expressed as a dose of polymer-bound drug. In the case of DX, only data obtained at optimal drug dose are presented.

antitumor drugs to anionic polyamino acids decreased in vitro cytotoxicity (8,9,18), our results suggest some differences in interaction of the drug-Py conjugate with tumor cells, as compared with other negatively charged macromolecular derivatives.

Although the antitumor activity per mole of bound drug was not increased (ie, a comparable activity was obtained with the same doses), treatment with the polymeric derivative was more effective than free drug because the reduced toxicity allowed an increase in drug dose. The best therapeutic effects were observed in the range of maximum tolerated doses, although treatment with DX-Py was characterized by a wide range of effective doses, especially when the drug was delivered ip.

The results presented in this paper show that with DX-Py we have been successful in preparing a polymeric derivative of DX with improved activity in several tumor systems. A similar result was obtained with poly-L-aspartic acid (8), thus suggesting the interest in anionic polymers as carriers for anthracyclines. Obviously, the precise mechanism of action of the DX-Py conjugate remains unclear. However, it is reasonable to assume that active drug is released from the polymer, as already observed with the DX-poly-L-aspartic acid (22), in which DX is linked to the carboxyl groups of the polyamino acid through the same ester bond. Preliminary studies indicated that free DX may be, at least in part, released in vivo following administration of the DX-Py conjugate. However, we cannot rule out the possibility that the intact drug-polymer conjugate contributes to the antitumor effects. Although this polymeric derivative of DX was designed assuming that the polymer would be biologically active (12) rather than just a simple source of slow release of the drug, the contribution of polymer-mediated mechanisms to improved activity of polymer-bound DX remains unclear. Both DX (23) and Py (24) are known as macrophage activators. This effect has been implicated in the antitumor activity of these agents (24,25). We have not determined whether DX-Py modified the interaction with these effector cells and therefore cannot comment on the contribution of this mechanism of action. However, since activated macrophages are recognized as an important component of host defenses against neoplastic growth and metastatic dissemination (26,27), this aspect of the mechanism of action of this polymeric derivative of the anthracyclines warrants further investigation.

The efficacy and the wide margin of safety of DX conjugated with this macromolecular carrier in the ip treatment of ascitic tumors suggest the potential interest in intracavitary chemotherapy. In this promising treatment approach, the use of DX is limited by local toxicity (28). This route of drug administration is of particular interest in the case of conjugates with immunomodulating macromolecules, since these compounds exert enhanced immunostimulating and antitumor effects when administered ip rather than iv (11,29). This may be the result of different distribution and compartmentalized macrophage activation using different routes of admin-

istration (30). Further pharmacologic and toxicologic studies on different experimental models of this polymeric derivative are needed to fully evaluate the therapeutic potential of this drug delivery system.

REFERENCES

1. DUNCAN R, and KOPEČEK J. Soluble synthetic polymers as potential drug carriers. Adv Polymer Sci 57:51-101, 1984.
2. POZNANSKY MJ, and JULIANO RL. Biological approaches to the controlled delivery of drugs: a critical review. Pharmacol Rev 36: 277-336, 1984.
3. ARNON R, and HURWITZ E. Antibody- and polymer-drug conjugates. *In* Targeted Drugs (Goldberg EP, ed). New York, John Wiley & Sons, 1983, pp 23-55.
4. ROWLAND GF. The use of antibodies in drug targeting and synergy. *In* Targeted Drugs (Goldberg EP, ed). New York, John Wiley & Sons, 1983, pp 57-72.
5. KATO Y, UMEMOTO N, KAYAMA Y, ET AL. A novel method of conjugation of daunomycin with antibody with a poly-L-glutamic acid derivative as intermediate drug carrier: an anti-α-fetoprotein antibody-daunomycin conjugate. J Med Chem 27:1602-1607, 1984.
6. ZAHARKO DS, PRZYBYLSKI M, and OLIVERIO VT. Binding of anticancer drugs to carrier molecules. Methods Cancer Res 16:347-380, 1979.
7. PRATESI G, SAVI G, PEZZONI G, ET AL. Poly-L-aspartic acid as a carrier for doxorubicin: a comparative in vivo study of free and polymer-bound drug. Br J Cancer. 1985. In press.
8. ZUNINO F, GIULIANI F, SAVI G, ET AL. Antitumor activity of daunorubicin linked to poly-L-aspartic acid. Int J Cancer 30: 465-470, 1982.
9. ZUNINO F, SAVI G, GIULIANI F, ET AL. Comparison of antitumor effects of daunorubicin covalently linked to poly-L-amino acid carriers. Eur J Cancer Clin Oncol 20:421-425, 1984.
10. OTTENBRITE RM. Structure and biological activities of some polyanionic polymers. *In* Anionic Polymeric Drugs (Donaruma LG, Ottenbrite RM, and Vogl O, eds). New York, John Wiley & Sons, 1980, pp 21-48.
11. REGELSON W. Clinical and research concepts regarding polyanions and starch microspheres. *In* Anionic Polymeric Drugs (Donaruma LG, Ottenbrite RM, and Vogl O, eds). New York, John Wiley & Sons, 1980, pp 303-325.
12. BRESLOW DS. Biologically active synthetic polymers. Pure Appl Chem 46:103-113, 1976.
13. BUTLER GB. Synthesis, characterization, and biological activity of pyran copolymers. *In* Anionic Polymeric Drugs (Donaruma LG, Ottenbrite RM, and Vogl O, eds). New York, John Wiley & Sons, 1980, pp 49-141.
14. PRZYBYLSKI M, FELL E, and RINGSDORF H. Syntheses and characterization of polymeric derivatives of the antitumor agent methotrexate. Makromol Chem 179:1719-1733, 1978.
15. RAMONAS LM, ERICKSON LC, KLESSE W, ET AL. Differential cytotoxicity and DNA cross-linking produced by polymeric and monomeric activated analogues of cyclophosphamide in mouse L1210 leukemia cells. Mol Pharmacol 19:331-336, 1981.
16. TARNOWSKI GS, RALPH P, and STOCK CC. Sensitivity to chemotherapeutic and immunomodulating agents of two mouse lymphomas and of a macrophage tumor. Cancer Res 39:3964-3967, 1979.
17. TALMADGE JE, KEY M, and HART IR. Characterization of a murine ovarian reticulum cell sarcoma of histiocytic origin. Cancer Res 41:1271-1280, 1981.
18. KATO Y, SAITO M, FUKUSHIMA H, ET AL. Antitumor activity of 1-β-D-arabinofuranosylcytosine conjugated with polyglutamic acid and its derivative. Cancer Res 44:25-30, 1984.
19. KAPLAN AM. Antitumor activity of synthetic polyanions. *In* Anionic Polymeric Drugs (Donaruma LG, Ottenbrite RM, and Vogl O, eds). New York, John Wiley & Sons, 1980, pp 227-253.
20. HIRANO T, RINGSDORF H, and ZAHARKO DS. Antitumor activity of monomeric and polymeric cyclophosphamide derivatives compared with in vitro hydrolysis. Cancer Res 40:2263-2267, 1980.
21. GROS L, RINGSDORF H, and SCHUPP H. Polymeric antitumor agents on a molecular and on a cellular level? Angew Chem Int Ed Engl 20:305-325, 1981.
22. MAZZONI A, GAMBETTA RA, TRAVE F, ET AL. Comparative distribution of free and poly-L-aspartic acid linked doxorubicin in MS-2 sarcoma bearing mice. Cancer Drug Deliv 3:163-172, 1986.
23. HASKILL JS. Adriamycin-activated macrophages as tumor growth inhibitors. Cancer Res 41:3852-3856, 1981.
24. LOVELESS SE, and MUNSON AE. Maleic vinyl ether activation of murine macrophages against lung-metastasizing tumors. Cancer Res 41:3901-3906, 1981.
25. SALAZAR D, and COHEN SA. Multiple tumoricidal effector mechanisms induced by Adriamycin. Cancer Res 44:2561-2566, 1984.
26. KEY ME. Macrophages in cancer metastases and their relevance to metastatic growth. Cancer Metastasis Rev 2:75-88, 1983.
27. FIDLER IJ. Macrophages and metastasis—a biological approach to cancer therapy: presidential address. Cancer Res 45:4714-4726, 1985.
28. MARKMAN M. Intracavitary chemotherapy. Crit Rev Oncol Hematol 3:205-233, 1985.
29. REGELSON W, and PARKER G. The routinization of intraperitoneal (intracavitary) chemotherapy and immunotherapy. Cancer Invest 41:29-42, 1986.
30. TALMADGE JE, MALUISH AE, COLLINS M, ET AL. Immunomodulation and antitumor effects of MVE-2 in mice. J Biol Response Mod 3:634-652, 1984.

Cell Kinetics as a Prognostic Marker in Locally Advanced Breast Cancer[1,2]

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The study analyzes the prognostic implication of cell kinetics on 52 locally advanced breast cancers. All patients were subjected to a multimodal treatment, including primary chemotherapy with doxorubicin and vincristine, surgery, or radiotherapy followed by additional chemotherapy with the same drug regimen. Pretreatment labeling index (LI) is not related to response to primary chemotherapy, whereas it provides information on the course of the disease. In fact, high LI significantly predicts a higher progression rate at the end of the entire combined treatment, a shorter time to disease progression, and a poorer probability of 4-year survival in comparison to low LI. From the present findings, LI appears to be a useful tool to select women with locally advanced breast cancer that would require very aggressive treatment. [Cancer Treat Rep 71:375-379, 1987]

In the last decade, morphologic, hormonal, and cell kinetic aspects of breast cancer have been extensively investigated to improve the knowledge of the natural history including the search for reliable prognostic markers. However, most of the studies were performed in women with early breast cancer and only rarely in patients with advanced disease. In the present study, we have investigated the prognostic implication of cell kinetics on locally advanced breast cancer. The labeling index (LI) has, in fact, proven to be a biologic marker of tumor aggressiveness affecting the clinical outcome of different human tumor types (1-3), including resectable breast cancer (4-7).

PATIENTS AND METHODS

Patient Population

The LI was determined in 52 women with locally advanced (T3b-T4) breast cancer. These patients belonged to a series of 162 consecutive women entered from September 1975 to September 1980 into a prospective randomized study (8). At that time cell kinetic determination was not routinely performed, and fresh material prior to start of treatment was available only for a few of a totally unselected series of patients. The therapeutic trial design can be summarized as follows. After three cycles of primary chemotherapy, patients with local tumor control (defined as absence of local tumor progression) and no overt distant metastases were either irradiated or subjected to mastectomy. Further chemotherapy was then administered after locoregional treatment. Primary chemotherapy consisted of doxorubicin (60 mg/m^2 on Day 1) and vincristine (1.2 mg/m^2 on Days 1 and 8) given iv every 21 days, for a total of three cycles. High-energy irradiation was delivered to the breast and regional lymph node-bearing areas to a total of 6000 rad plus a boost dose of 1000 rad to the breast. Surgery consisted of either Halsted or modified radical mastectomy. Six to seven additional cycles of the same drug combination were then administered.

Overall, main characteristics of the 52 women who had LI determination were not different from those of the main series. More than 90% of the patients were classified as T4, and 85% had clinically palpable axillary lymph nodes. Seventy-seven percent of the patients had tumors measuring > 5 cm in diameter on physical examination. Thirty-two patients (62%) were designated to receive radiotherapy as part of their combined treatment, and 20 women (38%) were allocated to undergo mastectomy. Pretreatment characteristics were evenly distributed between the two treatment groups with the exception of menopausal status. Most of the postmenopausal women in this series were in the radiotherapy arm (21 of 27, 78%), whereas 14 of 25 (56%) premenopausal patients belonged to the mastectomy group.

[1] Received Aug 26, 1986; revised Dec 10, 1986; accepted Jan 5, 1987.
[2] Supported in part by a grant from the Italian National Research Council, Special Project "Oncology," contract 86.02609.44.
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[4] We thank Miss L. Ventura and S. Canova for skilled technical assistance and Miss R. Ballarini for editing the manuscript.
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Study Parameters and Follow-up

In the absence of progressive disease, defined as either increase in tumor mass (at least 25% of the original size) or appearance of new disease manifestations at any time during treatment, response was categorized at the end of the entire program. Partial remission (PR) was defined as a reduction > 50% in the product of the two largest diameters, whereas complete remission (CR) was defined as a complete clinical and mammographic disappearance of the tumor mass. CR could have been achieved during or after the first three cycles of doxorubicin and vincristine but should have persisted for a minimum of 1 month from completion of all modalities.

Follow-up studies included measurement of involved sites on Day 1 of each treatment cycle during the initial chemotherapy phase, as well as prior to radiotherapy or surgery and 1 month after the end of radiotherapy. Roentgenograms and blood chemistry were repeated before either locoregional modality. Chest x-ray and mammography of the irradiated breast were then performed every 3 months, and bone x-ray, liver scan, and blood chemistry were repeated every 4–6 months. Complete physical examination was performed on Day 1 of each treatment cycle during the second part of chemotherapy and was repeated every 3 months thereafter.

Determination of LI

LI was determined on fresh tumor material immediately after the initial biopsy for histologic confirmation. Following removal of fat and necrotic areas, the neoplastic tissue was minced into fragments of a few cubic millimeters. From 15 to 20 fragments were placed in 199 medium with 20% fetal calf serum, antibiotics (100 units of penicillin and 100 μg/ml of streptomycin), and 6 μCi/ml of ^{3}H-thymidine (specific activity, 5 Ci/mmol) and incubated for 1 hour in agitation at 37°C. Media and serum were supplied by Flow Laboratories (Irvine, United Kingdom), and the radioactive precursor was purchased from the Radiochemical Centre (Amersham, United Kingdom). At the end of incubation, fragments were fixed in Bouin's solution, dehydrated in alcohol, and embedded in paraffin. Autoradiography was performed on histologic sections according to the stripping film (Kodak AR10) technique. After an exposure time of 10 days at 4°C, autoradiograms were developed in Kodak D 19b for 5 minutes at 18°C and fixed in Kodak F5. The samples were stained with hematoxylin and eosin at 4°C.

The LI was determined by scoring a total of 3000–10,000 cells from different specimens of the same tumor. When the specimens were small enough to allow the precursor to penetrate completely, counting was done through all sections. Otherwise, the counting was limited to the periphery of the section. No threshold for considering labeled nuclei was necessary because the background was always < 1.5 grains/100 μm^2, and silver grains of the background were therefore only occasionally observed above the nuclei. The minimum number of grains per nucleus of labeled cells was 20.

Since frequency distribution of LI values of primary breast cancer was exponential (9), the median LI value was used as cutoff to discriminate slowly and rapidly proliferating tumors.

Statistical Analysis

The differences in the values of tumor proliferative activity in the various clinical subsets were compared by means of the Wilcoxon rank-sum test, whereas the association between proliferative activity and objective clinical response was assessed by means of the kappa test. Freedom from progression (FFP) was considered as the interval between the start of treatment and the first documented evidence of treatment failure or the date of the last follow-up, whichever occurred first. Both FFP and overall survival were computed with the product limit estimate. The median time of observation for the overall series of patients was 41 months. The log-rank test (10) was used to assess the statistical significance of differences between the different subgroups. The percentage of progression-free or live patients was reported for one point in time (4 years) as derived from life-table plots.

RESULTS

The proliferative activity of the primary tumor in relation to various clinical characteristics is shown in table 1. No significant difference was found when tumor proliferative activity was analyzed in relation to menopausal status and type of locoregional treatment. When nodal status and primary tumor size, both assessed on clinical grounds, were examined, low LI was correlated with absence of palpable axillary nodes and small tumor sizes, while high LI values were related to palpable nodes and tumors > 5 cm in diameter.

The proliferative activity was not correlated with response to primary chemotherapy. In fact, 26 of 52 patients achieved PR following three treatment cycles. Of 26 patients with low LI, 13 achieved partial tumor response and the identical proportion (13 of 26) was observed in patients with high LI. By contrast, the proliferative activity of the primary tumor was able to discriminate between patients who showed progressive disease by the end of the entire combined treatment. In fact, 15 patients progressed while on treatment: four of 26 (15.4%) with low LI and 11 of 26 (42.3%) with high LI ($P = 0.032$).

Figure 1 shows the curves of FFP. Although at 4 years the curves of low versus high LI have a tendency to converge, the median time to disease progression was longer in women with slowly proliferating tumors compared to women with rapidly proliferating tumors (21 vs 10 months).

The overall survival is shown in figure 2. At 4 years from starting treatment, 68.1% of women with slowly

TABLE 1.—Relation between cell kinetics and clinical features

	No. of patients (%)	LI (%) Median	LI (%) Range	P value
Overall	52	3.6	0.01–19.1	—
Menopause				
Premenopausal	25 (48)	4.4	0.01–15.0	Not significant
Postmenopausal	27 (52)	3.1	0.5–19.1	
Tumor size (cm)				
< 5	12 (23)	1.9	0.01–10.4	
5–10	34 (65)	3.6	0.01–19.1	0.023
> 10	6 (12)	4.2	1.00–10.5	
Nodal status				
N_0	8 (15)	1.8	0.01–6.0	0.046
N_{1-2}	44 (85)	3.7	0.01–19.1	
Treatment arm				
Radiotherapy	32 (62)	3.1	0.01–19.1	Not significant
Surgery	20 (38)	4.8	0.01–17.0	

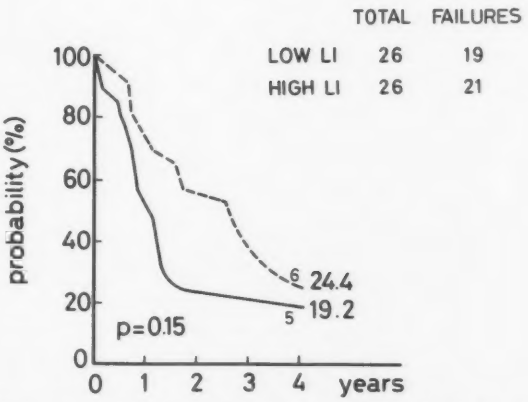

FIGURE 1.—Freedom from progression in patients with locally advanced breast cancer as a function of pretreatment proliferative activity. --- = slowly proliferating; —— = rapidly proliferating.

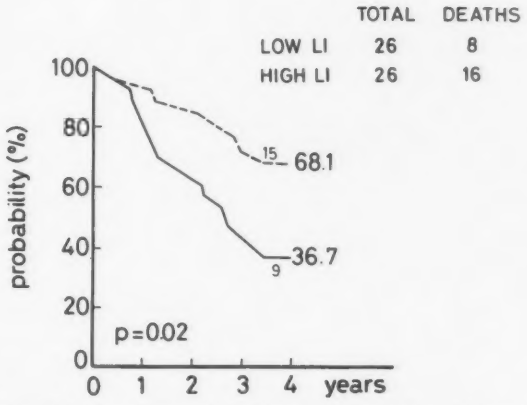

FIGURE 2.—Overall survival of patients with locally advanced breast cancer as a function of pretreatment proliferative activity. --- = slowly proliferating; —— = rapidly proliferating.

proliferating tumors were alive compared to 36.7% of women with rapidly proliferating tumors ($P = 0.02$). Within premenopausal patients, the proliferative activity failed to affect the overall results significantly, and the two curves started to diverge only after the second year (fig 3A). By contrast, in postmenopausal women the difference between high and low LI was significant ($P = 0.01$; fig 3B). Table 2 shows the 4-year survival as a function of LI in relation to the size of primary tumor and the nodal status. The small proportion of patients without clinically palpable axillary nodes (eight women) and with tumors up to 5 cm in diameter (12 women) prevented an adequate statistical analysis. The proliferative activity of the primary tumors was able to discriminate the 4-year total survival in women with palpable axillary nodes ($P = 0.006$). In patients presenting with tumors > 5 cm the difference between low and high LI was of borderline significance.

DISCUSSION

Locally advanced breast cancer (T3b-T4, Stage III) comprises a heterogeneous group of disease presentations whose definition varies from one study to another. Traditionally, locally advanced breast cancers have been considered incurable by locoregional therapies alone, either because patients present with technically inoperable tumors or because of biologic and strategic reasons based on the generally poor total survival (11). However, not all patients have an invariably poor prognosis. The fact that about 30% of women without inflammatory carcinoma remain alive at 5 years following locoregional treatment could not necessarily be attributed to a therapeutic effect but rather to the presence of subsets with favorable prognosis.

During the last 15 years, a number of uncontrolled studies were undertaken in locally advanced breast cancer utilizing various polydrug regimens or hormones followed by radiotherapy with or without subsequent surgery and further systemic therapy. The available

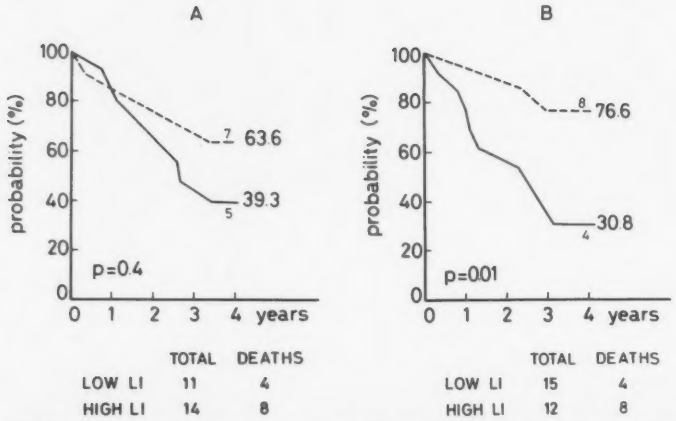

FIGURE 3.—Overall survival of pre- (A) and post- (B) menopausal patients with locally advanced breast cancer as a function of pretreatment proliferative activity. --- = slowly proliferating; —— = rapidly proliferating. *P* values are adjusted for therapy.

results (11,12) indicate that approximately 50%–80% of women will have objective response to primary chemotherapy, but the CR rate is < 20%, with the exception of patients with inflammatory carcinoma. It is still uncertain whether combination chemotherapy has significantly improved the 5-year survival which is now in the range of 45%–55%. Furthermore, it is not yet clear whether the use of primary chemotherapy improves local control.

The results of our study, in agreement with those recently published by Tubiana et al (5), indicate that in locally advanced breast cancer LI can identify two prognostic subsets with significantly different survival rates in relation to the proliferative activity of the primary neoplasm. Patients with tumors with low LI exhibit a 4-year survival superior to that of patients with tumors with high LI. The fact that LI was unable in this series to predict tumor response could probably be attributed to the moderately intensive chemotherapy regimen used. In fact, in this series of patients we administered a lower dose of doxorubicin (60 mg/m^2) compared to our previous series (75 mg/m^2) and the difference in dosage was reflected in a different response rate after three initial cycles (49% vs 62%). Moreover, it cannot be excluded that the lack of predictivity of proliferative rate for response to primary chemotherapy, in contrast to the data reported by Sulkes et al (13) on disseminated breast cancers, can be ascribed to the different types of drug combinations used.

TABLE 2.—Probability of 4-yr survival as a function of proliferative activity related to tumor size and nodal status

	Probability (%) of survival at 4 yrs		
	Low LI	High LI	*P* value
Overall series	68.1	36.7	0.02*
Tumor size (cm)			
≤ 5	71.4	50.0	
> 5	65.8	34.3	0.06
Nodal status			
N_0	66.7	100.0	
N_{1-2}	70.2	28.0	0.006

*Unadjusted; $P = 0.035$ adjusted for tumor size and nodal status.

In conclusion, as already observed for node-negative and -positive resectable tumors (4–7,9), in locally advanced noninflammatory breast cancer LI is an important indicator of biologic aggressiveness. Considering the tumor cell heterogeneity of breast cancer, particularly in the presence of large tumor masses, and the present uncertainty concerning improved survival following multimodality treatment, LI appears from the practical point of view to be a useful tool to better define women who would require very aggressive therapy.

REFERENCES

1. COSTA A, BONADONNA G, VILLA E, ET AL. Labeling index as a prognostic marker in non-Hodgkin lymphomas. JNCI 66:1-5, 1981.
2. DURIE BGM, SALMON SE, and MOON TE. Pretreatment tumor mass, cell kinetics, and prognosis in multiple myeloma. Blood 55: 364-372, 1980.
3. ELEQUIN FT, MUGGIA FN, GHOSSEIN NA, ET AL. Correlation between in vitro labeling indices (LIs) and tumor regression following radiotherapy. Int J Radiat Oncol Biol Phys 4:207-213, 1978.
4. MEYER JS, FRIEDMAN E, MCCRATE M, ET AL. Prediction of early course of breast carcinoma by thymidine labeling. Cancer 51:1879-1886, 1983.
5. TUBIANA M, PEJOVIC MH, CHAVAUDRA G, ET AL. The long-term prognostic significance of the thymidine labeling index in breast cancer. Int J Cancer 33:441-445, 1984.
6. SILVESTRINI R, DAIDONE MG, and GASPARINI G. Cell kinetics as a prognostic marker in node-negative breast cancer. Cancer 56:78-83, 1985.
7. SILVESTRINI R, DAIDONE MG, DI FRONZO G, ET AL. Prognostic implication of labeling index versus estrogen receptors and tumor size in node-negative breast cancer. Breast Cancer Res Treat 7: 161-169, 1986.
8. DE LENA M, VARINI M, ZUCALI R, ET AL. Multimodality treatment for locally advanced breast cancer. Results of chemotherapy-

radiotherapy versus chemotherapy-surgery. Cancer Clin Trials 4: 229-236, 1981.
9. GENTILI C, SANFILIPPO O, and SILVESTRINI R. Cell proliferation and its relationship to clinical features and relapse in breast cancers. Cancer 48:974-979, 1981.
10. PETO R, PIKE MC, ARMITAGE P, ET AL. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. Br J Cancer 35:1-39, 1977.
11. HENDERSON IC, HAYES DF, COME S, ET AL. New agents and new medical treatments for advanced breast cancer. Semin Oncol, 1986. In press.
12. VALAGUSSA P, ZAMBETTI M, BIGNAMI P, ET AL. T3b-T4 breast cancer: factors affecting results in combined modality treatments. Clin Exp Metastasis 1:191-202, 1983.
13. SULKES A, LIVINGSTON RB, and MURPHY WK. Tritiated thymidine labeling index and response in human breast cancer. JNCI 62:513-515, 1979.

Cytotoxicity of Floxuridine and 5-Fluorouracil in Human T-Lymphoblast Leukemia Cells: Enhancement by Leucovorin[1,2]

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The inhibitory effects of leucovorin (LV) combined with 5-fluorouracil (FUra) or floxuridine (FdUrd) on growth of human T-lymphoblast leukemia cells (CCRF-CEM) were determined as a function of time, dose, and sequence of exposure. Exposure of CCRF-CEM cells in exponential growth to LV (1–100 μM) for 4 hours and to FUra (100 μM) or FdUrd (0.5 μM) during the last 2 hours resulted in synergistic inhibitory effects on cell growth. Synergism was dependent on LV dose (100 > 10 > 1 μM) and did not occur at 0.1 μM. No clear dependence of synergy on sequence was observed with FUra and LV combinations. With LV and FdUrd combinations, synergism was dependent on sequence of exposure (LV + FdUrd and LV→FdUrd were synergistic, but FdUrd→LV was not). Thymidine (0.1 μM), added after drug treatment, substantially rescued CCRF-CEM cells from LV→FUra cytotoxicity. Concomitant hypoxanthine (100 μM) only partially protected CCRF-CEM cells from the toxicity of this combination. These results are consistent with the hypothesis that the mechanism by which LV potentiates fluoropyrimidine cytotoxicity is the enhancement of complex formation between thymidylate synthase and 5-fluorodeoxyuridylate, presumably as a consequence of an increase of intracellular levels of 5,10-methylenetetrahydrofolate generated from LV. Also, enhanced stability of this complex in the presence of high levels of the folate coenzyme may contribute to the synergy observed. These data also provide a rationale for use of FUra and especially FdUrd and LV in the treatment of lymphoid malignancies in man. [Cancer Treat Rep 71:381–389, 1987]

5-Fluorouracil (FUra) is a clinically useful agent for the treatment of carcinoma of the gastrointestinal tract, breast, ovary, and head and neck (1), although its activity as a single agent in the disease is modest. FUra has so far been disappointing in the treatment of leukemias and lymphomas. It has been suggested, however, that it might also have definite, although limited, single-agent activity in acute leukemia in adults (2).

FUra produces its cytotoxic effects via metabolic conversion to the active nucleotide derivatives FUTP, FdUMP, and FdUTP (3). FUTP is incorporated into all species of RNA and may interfere with its synthesis or function (4,6), with consistent effects on cell survival (7); FdUTP is incorporated into DNA by α polymerase and is rapidly removed by uracil-*N*-glycosylase (8,9), leading to possible lethal effects (10); and FdUMP binds tightly to dTMP synthase in the presence of the coenzyme 5,10-methylenetetrahydrofolate ($5,10\text{-}CH_2FH_4$) (11,12), thus inhibiting dTMP formation and ultimately DNA synthesis. Although the ternary complex among the dTMP synthase, FdUMP, and $5,10\text{-}CH_2FH_4$ is a covalent one, nevertheless it does dissociate in the absence of excess of the folate coenzyme (13). Since this process occurs intracellularly the released enzyme would be protected from further FdUMP binding by expanded deoxyuridylate pools (14) with consequent resistance to FUra kill.

In recent years, it has been shown that appropriate modulation of FUra metabolic pathways in tumor cells by a second drug may selectively alter the intracellular pharmacokinetics and pharmacodynamics of the fluoropyrimidine, and hence may enhance its therapeutic efficacy even in the circumstance in which the drug has little single-agent activity (15).

[1] Received July 8, 1986; revised Dec 15, 1986; accepted Jan 12, 1987.

[2] Supported by Public Health Service grant CA-08010 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. E. Mini is a Leukemia Society of America Special Fellow and B. A. Moroson is an American Cancer Society Professor of Medicine and Pharmacology.

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[5] We thank Dawne Newcombe and Arlene Cashmore for their assistance in the preparation of the manuscript and illustrations.

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In vitro studies have shown that leucovorin (LV) can markedly enhance the activity of FUra and its nucleotide floxuridine (FdUrd) against tumor cells of various origin (16,18), including murine leukemia cells. This enhancement of cytotoxicity is presumably the result of an increase in the intracellular pools of the reduced folate cofactors, including 5,10-CH_2FH_4, leading to an increased formation of the complex between dTMP synthase and FdUMP (17,19,20) and stabilization of this ternary complex (16,21).

These results indicate that endogenous folate cofactor levels in tumors only marginally sensitive to FUra may be sufficient for optimal growth but not for maximal inhibition of dTMP synthase by FdUMP and that the possibility exists in cancer patients to maximize this FUra DNA-directed effect by the use of an exogenous source of folate cofactors. Initial clinical studies using high doses of LV and FUra, initiated on the basis of these laboratory data, produced encouraging results in advanced gastrointestinal tumors, about double those expected from FUra treatment alone (50% and 37% response rates in previously untreated gastric and colorectal cancer patients, respectively) (22-27). Objective responses (18%) were also noted in colorectal cancer patients refractory to previous FUra treatment.

The purpose of this study was to examine the cytotoxic effects of LV and FUra or FdUrd on human leukemia T-lymphoblast cells (CCRF-CEM) in culture, during simultaneous or sequential exposure, and to determine the effects of this folate on the sites of action of fluoropyrimidines by modulating the cytotoxic effects of their combinations with interfering metabolites [ie, thymidine (dThd) and hypoxanthine (Hyp)]. Our results demonstrate that LV greatly enhances the growth-inhibitory effects of FUra and FdUrd against these cells. Observed synergism can be reversed by dThd rescue, while it is only partially reversed by Hyp protection. The relevance of these data to the mechanism of interaction is discussed (28).

MATERIALS AND METHODS

Chemicals.—LV, FUra, FdUrd, Hyp, and dThd were purchased from Sigma Chemical Co, St. Louis, MO. Media, sera, and antibiotics for cell culture were obtained from Grand Island Biological Co, NY, and plastic ware was obtained from Corning Glass Works, NY.

Cell line.—CCRF-CEM cells were from a cloned subline of the human T-lymphoblast cell line described by Foley et al (29). Cells were maintained as suspension cultures in RPMI-1640 medium supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μM/ml) at 37°C in a 5% CO_2 atmosphere and subcultured twice a week. Under these conditions the doubling time of exponentially growing cells was 20 hours. Cells were tested periodically using the Hoechst Stain Kit (Flow Laboratories, Inc, Rockville, MD) (30) and found to be mycoplasma-free.

Cell growth inhibition studies.—Exponentially growing cells were exposed to drugs at an initial density of about 2×10^5 cells/ml. All drugs were dissolved in water and prepared freshly before each experiment. After drug treatment, cells were harvested by centrifugation, washed twice, and resuspended in drug-free medium supplemented with 10% horse serum at a concentration of about 2×10^4 cells/ml. Subsequent changes in cell number were followed over a 5–7-day period using a Coulter counter (Model B, Coulter Electronics, Inc, Hialeah, FL). To compare the effects of drug treatment on cell growth suspended at a slightly different initial density, the cell numbers were converted to total cell divisions (TCD) calculated from the formula:

$$TCD = \frac{\log_{10}\ (N^t/n^0)}{\log_{10}2},$$

where n^0 is the initial cell number following resuspension and N^t is the cell number at time t. Growth curves were generated plotting TCDs as a function of time (31).

RESULTS

The inhibitory effects of LV, fluoropyrimidines, and their combinations on cell growth were determined by measurements of cell regrowth after washing and resuspension in drug-free medium following drug treatment, as described in the Materials and Methods section. LV used in this investigation was the commercially available form produced by a chemical reduction of folic acid, thus containing equal amounts of the active (*l*) and the inactive (*d*) diastereoisomers. Reported LV concentrations refer to the total diastereoisomer mixture (*dl*) of the folate.

Exposure of various durations (4 hours to continuous) to LV at concentrations between 0.01 and 100 μM did not produce any significant change in the growth of CCRF-CEM cells. When CCRF-CEM cells were exposed to LV (1, 10, and 100 μM) for 4 hours and to FUra (50, 100, and 250 μM) during the last 2 hours of exposure to the reduced folate, synergistic inhibitory effects on cell growth were observed. Maximum synergism occurred at 100 μM FUra (fig 1). Observed synergism was dependent on LV concentrations used ($100 > 10 > 1$ μM). Synergism did not occur at 0.1 μM LV (data not shown). When FdUrd was substituted for FUra at a concentration of 0.5 μM (approximately as toxic as 100 μM FUra) in the previously described sequential schedule (LV, 4 hours; fluoropyrimidine, last 2 hours), marked synergistic inhibitory effects on cell growth were noted (fig 2). At lower (0.1 μM) or higher (1 μM) concentrations of FdUrd, a lower degree of synergy was observed (data not shown). Dose-dependent synergism occurred at LV concentrations ≥ 1 μM. Degree of synergy was greater for LV-FdUrd as compared to LV-FUra sequential treatment.

To further test the sequence dependence of LV-fluoropyrimidine synergism, CCRF-CEM cells were exposed for 4 hours in sequence or simultaneously to LV (1, 10, and 100 μM) and to either FUra (100 μM) or FdUrd (0.1 μM) [ie, LV→FUra (or FdUrd); FUra (or FdUrd)

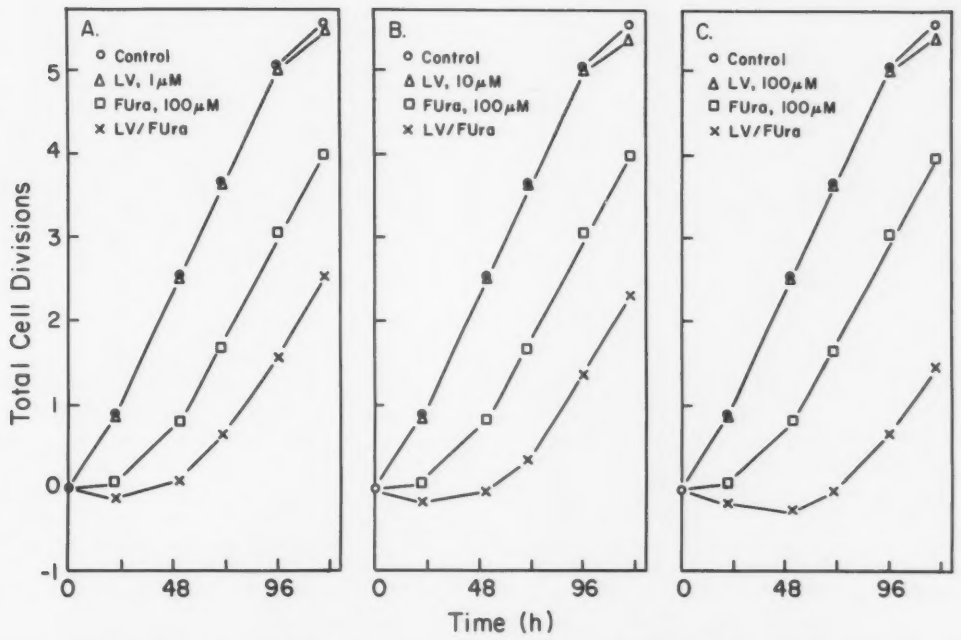

FIGURE 1.—Inhibitory effects of sequential LV-FUra treatments on growth of CCRF-CEM cells. Cells at a density of about 2×10^5/ml were exposed to LV (Δ) (Panel A, 1 μM; Panel B, 10 μM; Panel C, 100 μM) for 4 hrs and to FUra (100 μM) alone (□) or in combination (X) for the last 2 hrs of folate exposure. After drug exposure, cells were washed twice, resuspended in drug-free medium at a density of about 2×10^4/ml, and their growth followed. Growth curves of control (○) and treated cells were generated by plotting TCDs vs time. TCDs were calculated as described in the Materials and Methods section. The data represent the mean of duplicate samples from a single representative experiment.

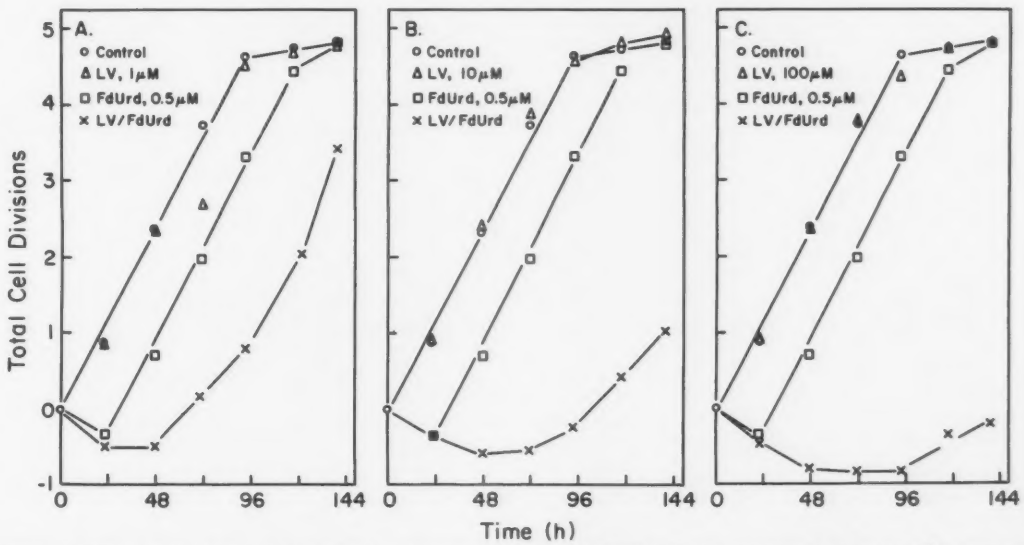

FIGURE 2.—Inhibitory effects of sequential LV-FdUrd treatments on growth of CCRF-CEM cells. Cells at a density of about 2×10^5/ml were exposed to LV (Δ) (Panel A, 1 μM; Panel B, 10 μM; Panel C, 100 μM) for 4 hrs and to FdUrd (0.5 μM) alone (□) or in combination (X) for the last 2 hrs of folate exposure. After drug exposure, cells were washed twice, resuspended in drug-free medium at a density of about 2×10^4/ml, and their growth followed. Growth curves of control (○) and treated cells were generated by plotting TCDs vs time. TCDs were calculated as described in the Materials and Methods section. The data points represent the mean of duplicate samples from a single representative experiment.

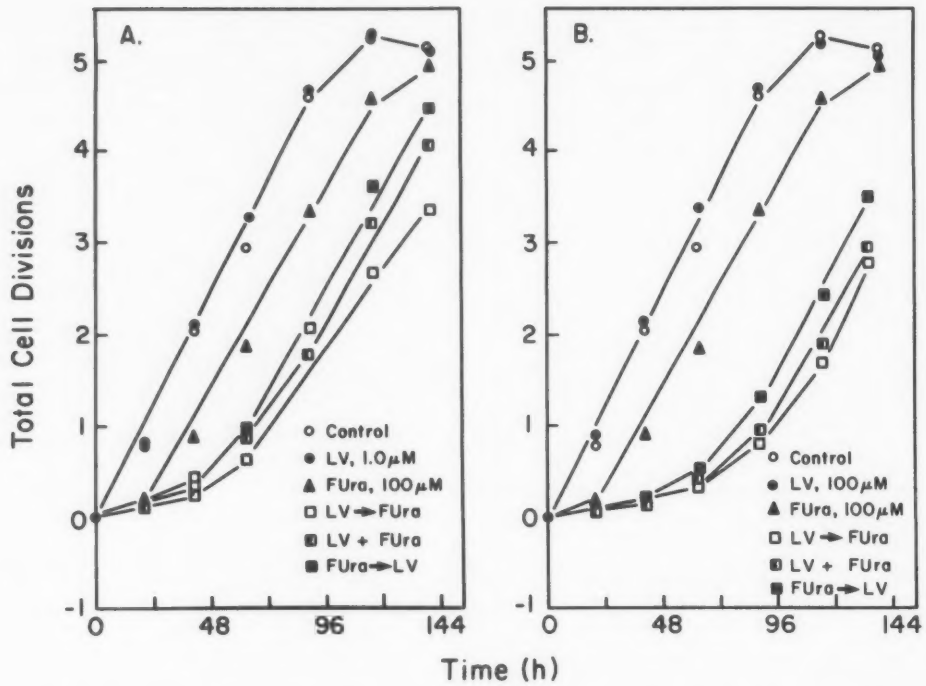

FIGURE 3.—Sequence-dependent effects of LV and FUra on CCRF-CEM cell growth. Cells at a density of about 2×10^5/ml were exposed to either FUra (100 μM) or LV (1 or 100 μM). Each drug exposure either alone or in simultaneous or sequential combination was for 4 hrs. After 4 hrs of exposure to the first drug, cells were washed twice and re-exposed to the second drug at the same density. After the final wash, cells were resuspended in drug-free medium at a density of about 2×10^4/ml and their growth was followed. Growth curves of control and treated cells were generated by plotting TCDs vs time. TCDs were calculated as described in the Materials and Methods section. Results represent mean values of duplicate determinations from a single representative experiment. ○ = control; ▲ = FUra; ● = LV (Panel A, 1 μM; Panel B, 100 μM), □ = LV→FUra; ◧ = LV + FUra; and ■ = FUra→LV.

→LV; LV + FUra (or FdUrd)]. Synergistic inhibitory effects on cell growth were observed with LV-FUra combinations, dependent on LV used (100 > 10 > 1 μM) (fig 3). The data with LV at 10 and 1 μM are not shown.

No clear dependence of synergism on sequence of exposure was observed. However, while the administration of LV followed by FUra (LV→FUra) and the simultaneous exposure of cells to folate and fluoropyrimidine (LV + FUra) obtained maximum inhibitory effects on cell growth, the reverse sequence (FUra→LV) obtained slightly less synergy. Synergy was also obtained with LV-FdUrd combinations (fig 4). It appeared to be dependent on the dose of LV used (100 > 10 > 1 μM) and on sequence of administration (the data with 10 and 1 μM are not shown). The degree of synergy was greater with the sequence LV-FdUrd as compared to that of the sequence LV→FdUrd; no synergy was observed using the sequence FdUrd→LV.

It is generally accepted that (*d*)-LV is devoid of activity as a folate coenzyme (32). The biological effects of this unnatural isomer, however, are still unknown. If, as a consequence of high intracellular levels of (*d*)-LV, possibly reached after exposure to high concentrations of the epimeric diastereoisomers (*dl*) of this folate, inhibition of enzymes involved in purine biosynthesis occurred, an increase in intracellular phosphoribosylpyrophosphate (PRPP) levels would result with consequent enhanced FUra activation via orotate phosphoribosyl transferase (33), ultimately leading to increased drug cytotoxicity. To prevent an eventual PRPP increase, cells were exposed to Hyp (1, 10, and 100 μM), a preformed purine base which requires PRPP as the phosphoribose donor for nucleotide formation (34-46), for 4 hours concomitantly with LV (10 μM) to prevent potentiation of cytotoxicity of subsequently administered FUra (2 hours, 100 μM). As shown in figure 5, Hyp up to 100 μM only partially protected CCRF-CEM cells from toxicity of the combination LV → FUra.

In contrast, dThd (0.1 μM) added to medium after drug treatment substantially rescued CCRF-CEM cells from the cytotoxicity of sequential LV→FUra (LV, 10 μM, 4 hours, followed by FUra, 100 μM, 2 hours), while it did not rescue these cells from the cytotoxic effects of FUra alone (fig 6). Higher concentrations of dThd (1 μM), however, were also able to rescue CCRF-CEM cells from the cytotoxicity of FUra alone (data not shown).

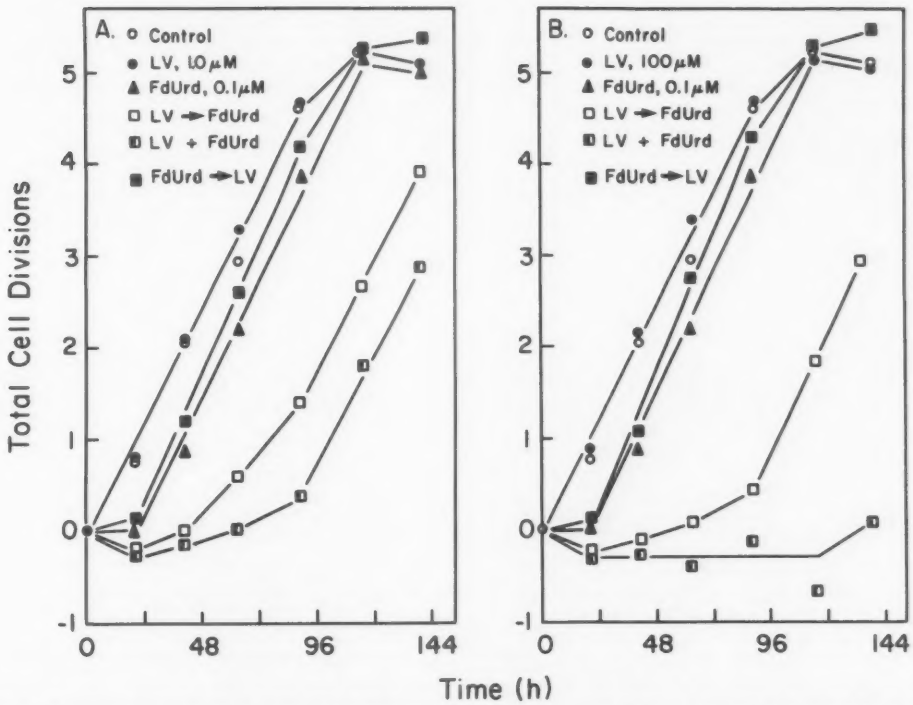

FIGURE 4.—Sequence-dependent effects of LV and FdUrd on CCRF-CEM cell growth. Cells at a density of about 2×10^5/ml were exposed to FdUrd (0.1 μM) or LV (1 or 100 μM). Each drug exposure either alone or in simultaneous or sequential combination was for 4 hrs. After 4 hrs of exposure to the first drug, cells were washed twice and re-exposed to the second drug at the same density. After the final wash, cells were resuspended in drug-free medium at a density of about 2×10^4/ml and their growth was followed. Growth curves of control and treated cells were generated by plotting TCDs vs time. TCDs were calculated as described in the Materials and Methods section. Results represent mean values of duplicate determinations from a single representative experiment. ○ = control; ▲ = FUra; ● = LV (Panel A, 1 μM; Panel B, 100 μM); □ = LV→FUra; ◧ = LV + FUra; and ■ = FUra→LV.

DISCUSSION

Although the cytotoxicity of FUra may result from different biochemical events, including incorporation of fluoronucleotides into RNA (4–7) and DNA (8–10), inhibition of dTMP synthase by FdUMP appears to be the most critical determinant of the action of this drug in most mouse and some human tumors.

On the basis of previous reports suggesting that responsiveness of human tumors to FUra and FdUrd is limited by the availability of the intracellular folate cofactor 5,10-CH_2FH_4, necessary for tight binding between dTMP synthase and FdUMP (16–21), we attempted to increase the marginal cytotoxic effects of these fluoropyrimidines against human T-lymphoblast leukemia cells (CCRF-CEM) in culture using an exogenous reduced folate, LV. Intracellularly, LV equilibrates with the other folate pools, including 5,10-CH_2FH_4 (32), which, in the form of polyglutamate derivatives, appears to be the actual cofactor for folate-dependent enzymes (41).

Although there are a variety of agents more useful than fluoropyrimidines for the treatment of leukemias and lymphomas, it would nevertheless be highly desirable to develop more effective drug combinations for these malignancies, where a considerable portion of patients either never respond or eventually relapse.

A short pretreatment (2 hours) with LV over a large range of concentrations (1–100 μM) enhanced the inhibitory effects of both FUra and FdUrd administered for the following 2 hours at approximately equitoxic concentrations (100 and 0.5 μM, respectively) on CCRF-CEM cell growth (figs 1 and 2). These data are in agreement with previous results of other investigators demonstrating that synergism occurs only in the presence of high extracellular LV levels (20 μM) in a variety of tumor cell types (16–18). These experiments, as well as ours, were conducted on cells cultured at folic acid concentrations allowing optimal growth (2.3–2.8 μM). Extensive analysis of codependence of synergism, however, has not yet been reported. Our data clearly demon-

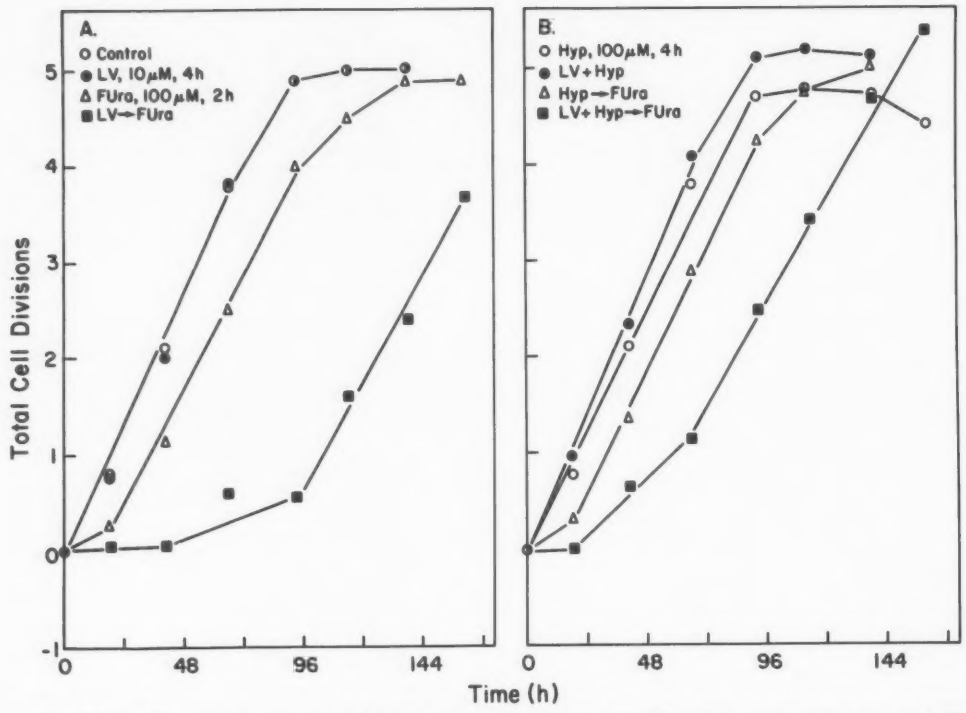

FIGURE 5.—Effects of Hyp protection on cytotoxicity of the sequential combination LV→FUra. Cells at a density of about 2×10^5/ml were first exposed to LV (10 μM) (●) or no drug (○) for 4 hrs in the absence or presence of Hyp (100 μM) (Panels A and B, respectively). After washing in drug-free medium, cells were exposed at the same density to FUra (100 μM) for 2 hrs following either no treatment (△) or LV (■). After final wash, cells were resuspended in drug-free medium at a density of about 2×10^4/ml and their growth was followed. Growth curves of control and treated cells were generated by plotting TCDs vs time. TCDs were calculated as described in the Materials and Methods section. Results represent mean values of duplicate determinations in a single representative experiment.

strate the direct dependence of the synergism on the dose of the folate used, in that the maximum effect occurred at 100 μM LV and decreased with decreasing LV concentrations (10 > 1 μM) until disappearance at 0.1 μM.

Exposure to LV at concentrations between 0.01 and 100 μM did not produce any significant change in the growth of CCRF-CEM cells maintained in complete culture medium containing 2.3 μM folic acid, a concentration optimal for cell growth.

Synergistic inhibitory effects on cell growth were observed when LV (1-100 μM, 4 hours) preceded FUra (100 μM, 4 hours) or FdUrd (0.1 μM, 4 hours) administration (LV→FUra, LV→FdUrd) or when the folate and one of the fluoropyrimidines were given simultaneously (LV + FUra, LV + FdUrd, 4 hours) (figs 3 and 4). A slightly lower degree of synergism was noted when FUra was given prior to LV (FUra→LV) (fig 3), while no synergism occurred when FdUrd was substituted for FUra in this reverse sequence schedule (FdUrd→LV) (fig 4). Also, the degree of synergism was higher for LV-FdUrd combinations than for LV-FUra combinations.

These differences in the degree and sequence-dependence of synergism might be explained by the different rates of conversion of the two fluoropyrimidines to the common active metabolite FdUMP. In CCRF-CEM cells, FdUrd will presumably reach higher FdUMP levels in a shorter time as compared to FUra. As described by Piper and Fox (42) and confirmed in our laboratory (data not shown), these cells lack uridine and dThd phosphorylases. Thus, FdUrd is rapidly and exclusively converted to FdUMP via dThd kinase, while FUra is only partially converted to the fluorodeoxynucleotide via a multienzymatic pathway including orotate phosphoribosyl transferase and ribonucleotide reductase, which presumably may require a longer time.

Exposure after drug treatment to dThd (0.1 μM), the pyrimidine nucleotide that circumvents the block of dTMP synthesis induced by FdUMP, substantially reverted the synergistic effects of the combination LV-FUra (fig 6), but did not relieve the cytotoxicity of FUra alone. This rescue experiment suggested that the prolonged inhibition of dTMP synthase by FdUMP is the primary event responsible for the enhancement of FUra cytotoxicity by LV.

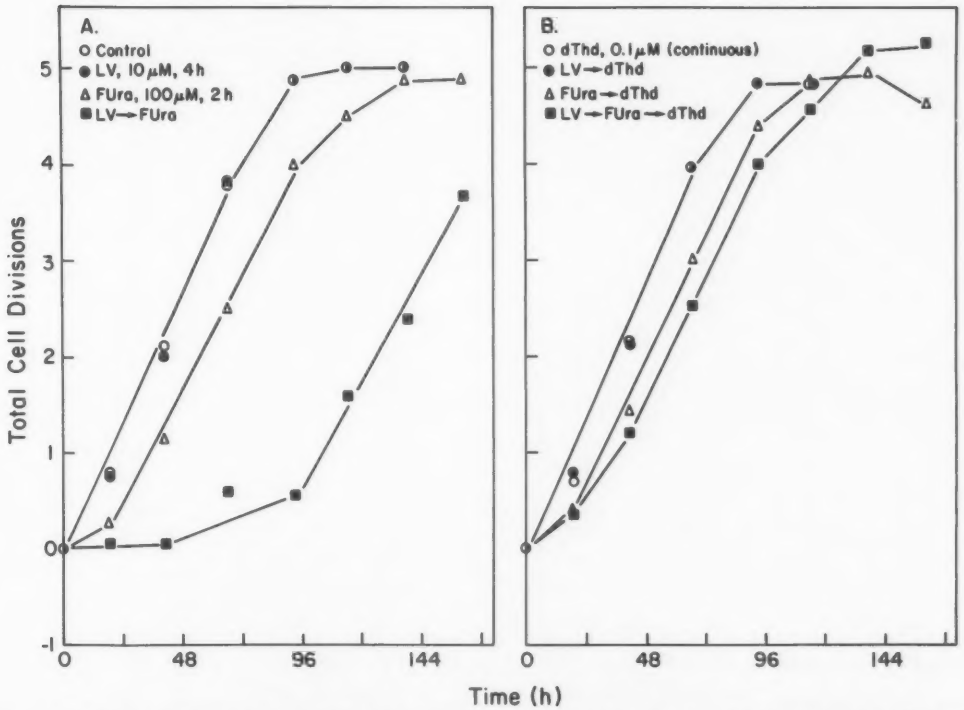

FIGURE 6.—Effects of dThd rescue on cytotoxicity of the sequential combination LV→FUra. Cells at a density of about 2×10^5/ml were first exposed to LV (10 μM) (●) or no drug (○) for about 4 hrs. After washing in drug-free medium cells were then exposed at the same density to FUra (100 μM) for 2 hrs following either no treatment (Δ) or LV (■). After final wash, cells were resuspended in drug-free medium with or without dThd (0.1 μM) (Panels B and A, respectively) at a density of about 2×10^4/ml and their growth was followed. Growth curves of control and drug-treated cells were generated by plotting TCDs vs time. TCDs were calculated as described in the Materials and Methods section. Results represent mean values of duplicate determinations from a single representative experiment.

Synergistic inhibitory effects on CCRF-CEM cell growth were also obtained when another folate coenzyme, 5-methyltetrahydrofolate (5-CH_3FH_4), was administered at equimolar concentrations in place of LV with both fluoropyrimidines in the described simultaneous and sequential schedules (data not shown).[6] It is known that this reduced folate is also converted intracellularly to tetrahydrofolate via methionine synthetase in the presence of homocysteine and B_{12} and then to 5,10-CH_2FH_4 (32). (*l*)-5-CH_3FH_4 is the folate to which (*l*)-LV is rapidly converted following in vivo administration (43–45). Because of these pharmacokinetic patterns, the demonstration of the favorable interactions of 5-CH_3FH_4 and fluoropyrimidines at the cellular level is of critical importance in view of the clinical use of LV and fluoropyrimidine combinations.

Although it is generally accepted that only the natural (*l*) isomer of LV is active as a folate coenzyme (32), the biochemical and biological effects of the unnatural (*d*) isomer are not yet well known. It has previously been shown that the unnatural (*d*) isomer was not able to utilize the specific membrane carrier system for reduced folates and 2,4-diamino analogs as effectively as the natural (*l*) isomer (46). Intracellularly, (*d*)-LV might, for instance, enhance FUra metabolism via orotate phosphoribosyl transferase by increasing PRPP intracellular levels as a consequence of inhibition of folate-dependent enzymes in the purine pathway. To test this possible interaction, we exposed cells concomitantly with LV to Hyp, a preformed purine base which competes with FUra for the common cosubstrate for nucleotide formation, the phosphoribose donor PRPP (34–36). This would have prevented potentiations of subsequently administered FUra. We were able to show only slight protection of LV-FUra cytotoxicity by Hyp at concentrations up to 100 μM (fig 5).

The observation that intermittent or continuous exposure of CCRF-CEM cells to (*d*)-LV concentrations up to 100 μM did not produce any inhibition on cell growth in our experimental conditions also indirectly demonstrated that the ability of (*d*)-LV of being transported intracellularly and interfering with folate-dependent metabolism was poor.

[6] Mini E, Mazzei T, Coronnello M, et al. Manuscript in preparation.

In conclusion, these data are consistent with the hypothesis that LV-fluoropyrimidine synergism is a consequence of enhanced covalent binding between dTMP synthase and FdUMP (17,19,20) and of decreased dissociation of this complex (16,21) in the presence of increased levels of the folate coenzyme 5,10-CH_2FH_4 generated intracellularly from LV. At concentrations of LV tested in this investigation, other possible effects of the folate on intracellular pharmacokinetics and pharmacodynamics of fluoropyrimidines appear to be of minor relevance to synergism.

The data encourage further cytotoxicity and biochemical studies using both human leukemic and normal bone marrow cells on the mechanism of synergism, differential sensitivity, and optimal dose scheduling prior to clinical use. Based on these in vitro results, studies of combinations of fluoropyrimidines with either LV or 5-CH_3FH_4 are also warranted in leukemia and lymphoma patients.

NOTE ADDED IN PROOF

Since the submission of these studies, a paper has been published in *Cancer Research* (46:5229-5235, 1986) by K. Keyomarsi and R. Moran that comes to essentially the same conclusions reached in the present manuscript.

REFERENCES

1. TAPAZOGLOU E, KISH J, ENSLEY J, ET AL. The activity of a single-agent 5-fluorouracil infusion in advanced and recurrent head and neck cancer. Cancer 57:1105-1109, 1986.
2. COSTANZI JJ, GAGLIANO R, COLTMAN CA, JR, ET AL. 5-Fluorouracil in the treatment of acute leukemia: a Southwest Oncology Group study. Cancer Treat Rep 63:2126-2128, 1979.
3. HEIDELBERGER C, DANENBERG PV, and MORAN RG. Fluorinated pyrimidines and their nucleosides. Adv Enzymol 54:57-119, 1983.
4. WILKINSON DS, and PITOT HC. Inhibition of ribosomal ribonucleic acid maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. J Biol Chem 248:63-68, 1973.
5. GLAZER RI, and HARTMAN FD. The effects of 5-fluorouracil on the synthesis and methylation of low molecular weight nuclear RNA in L1210 cells. Mol Pharmacol 17:245-249, 1980.
6. GLAZER RI, and HARTMAN FD. Analysis of the effect of 5-fluorouracil on the synthesis and translation of polysomal poly(A) RNA from Ehrlich ascites cells. Mol Pharmacol 19:117-121, 1981.
7. GLAZER RI, and LLOYD LS. Association of cell lethality with incorporation of 5-fluorouracil and 5-fluorouridine into nuclear RNA in human colon carcinoma cells in culture. Mol Pharmacol 21:468-473, 1982.
8. CARADONNA SJ, and CHENG C. The role of deoxyuridine triphosphate nucleotidohydrolase, uracil-DNA glycosylase, and DNA polymerase alpha in the metabolism of FUdR in human tumor cells. Mol Pharmacol 18:513-520, 1980.
9. INGRAHAM HA, TSENG BY, and GOULIAN M. Mechanism for exclusion of 5-fluorouracil from DNA. Cancer Res 40:998-1001, 1980.
10. KUFE DW, SCOTT P, FRAM R, ET AL. Biological effect of 5-fluorodeoxyuridine incorporation in L1210 deoxyribonucleic acid. Biochem Pharmacol 32:1337-1340, 1983.
11. SANTI DV, MCHENRY CS, and SOMMER H. Mechanism of interaction of thymidylate synthetase with 5-fluoro-deoxyuridylate. Biochemistry 13:471-481, 1974.
12. DANENBERG PV, LANGENBACH RJ, and HEIDELBERGER C. Structures of irreversible complexes of thymidylate synthetase and fluorinated pyrimidine nucleotides. Biochemistry 13:926-933, 1974.
13. LOCKSHIN A, and DANENBERG PV. Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. Biochem Pharmacol 30:247-257, 1981.
14. MORAN RG, SPEARS CP, and HEIDELBERGER C. Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthetase. Proc Natl Acad Sci USA 76:1456-1460, 1979.
15. BERTINO JR, and MINI E. Does modulation of 5-fluorouracil by metabolites or antimetabolites work in the clinic? *In* New Avenues in Developmental Cancer Chemotherapy. Proceedings of the 8th Annual Bristol-Meyers Symposium on Cancer Research, 1986. In press.
16. EVANS RM, LASKIN JD, and HAKALA MT. Effects of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288-3295, 1981.
17. ULLMAN B, LEE M, MARTIN DW, ET AL. Cytotoxicity of 5-fluoro-2′-deoxyuridylate: requirement for reduced folate cofactors and antagonism by methotrexate. Proc Natl Acad Sci USA 75:980-983, 1978.
18. WAXMAN S, and BRUCKNER H. The enhancement of 5-fluorouracil antimetabolic activity by leucovorin, mandione and α-tocopherol. Eur J Cancer Clin Oncol 18:685-692, 1982.
19. HOUGHTON JA, MARODA SJ, PHILLIPS JO, ET AL. Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinoma in mice. Cancer Res 41:144-149, 1981.
20. HOUGHTON JA, SCHMIDT C, and HOUGHTON PJ. The effects of derivatives of folic acid on the fluorodeoxyuridylate thymidylate synthetase covalent complex in human colon xenografts. Eur J Cancer Clin Oncol 18:347-354, 1982.
21. YIN MB, ZAKRZEWSKI SF, and HAKALA MT. Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. Mol Pharmacol 23:190-197, 1983.
22. BERTRAND M, DOROSHOW JH, MULTHAUF P, ET AL. Combination chemotherapy with high-dose continuous infusion leucovorin and bolus 5-fluorouracil in patients with advanced colorectal cancer. *In* Advances in Cancer Chemotherapy (Bruckner HW, and Rustum YM, eds). New York, John Wiley and Sons Inc, 1984, pp 73-76.
23. BUKOWSKI RM, and CUNNINGHAM J. Efficacy of 5-fluorouracil in combination with leucovorin in patients with colorectal carcinoma: continuous infusion of low doses. *In* Advances in Cancer Chemotherapy (Bruckner HW, and Rustum YM, eds). New York, John Wiley and Sons Inc, 1984, pp 69-72.
24. BYRNE PJ, TREAT J, MCFADDEN M, ET AL. Therapeutic efficacy of the combination of 5-fluorouracil and high-dose leucovorin in patients with advanced colorectal carcinoma: single daily intravenous dose for five days. *In* Advances in Cancer Chemotherapy (Bruckner HW, and Rustum YM, eds). New York, John Wiley and Sons Inc, 1984, pp 65-67.
25. MACHOVER D, SCHWARZENBERG L, GOLDSCHMIDT E, ET AL. Treatment of advanced colorectal and gastric adenocarcinomas with 5-FU combined with high-dose folinic acid: a pilot study. Cancer Treat Rep 66:1803-1807, 1982.
26. MADAJEWICZ S, PETRELLI N, RUSTUM YM, ET AL. Phase I-II trial of high-dose calcium leucovorin and 5-fluorouracil in advanced colorectal cancer. Cancer Res 44:4467-4469, 1984.
27. BRUCKNER HW, ROBOZ J, SPIGELMAN M, ET AL. An efficient leucovorin-5-fluorouracil sequence: dosage escalation and pharmacologic monitoring. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil-Leucovorin in Calcium Combination (Bruckner HW, and Rustum YM, eds). New York, John Wiley and Sons Inc, 1984, pp 49-54.
28. MINI E, MOROSON BA, and BERTINO JR. Leucovorin (LV)-fluoropyrimidine synergy in the human leukemic lymphoblast cell line CCRF-CEM. Proc Am Assoc Cancer Res 26:324, 1985.
29. FOLEY GE, LAZARUS H, FARBER S, ET AL. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 18:522-529, 1965.
30. CHEN TR. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp Cell Res 104:255-

262, 1977.
31. MINI E, MOROSON BA, CASHMORE AR, ET AL. Synergistic effects of sequential methotrexate and 5-fluorouracil treatment on the growth of human B- and T-lymphoblast cell lines: modulation by horse and fetal bovine serum. *In* Proceedings of the 13th International Congress of Chemotherapy (Spitzy KH, and Karrer K, eds). Vienna, Egermann Verlag, 1983, part 252, pp 5–8.
32. BLAKLEY RL. *In* The Biochemistry of Folic Acid and Related Pteridines. New York, American Elsevier Publishing Co, 1969.
33. REYES P. The synthesis of 5-fluorouridine 5′-phosphate by a pyrimidine phosphoribosyltransferase of mammalian origin. I. Some properties of the enzyme from P1534J mouse leukemic cells. Biochemistry 8:2057–2062, 1969.
34. ULLMAN B, and KIRSCH J. Metabolism of 5-fluorouracil in cultured cells: protection from 5-fluorouracil cytotoxicity by purines. Mol Pharmacol 15:357–366, 1979.
35. YOSHIDA M, and HOSHI A. Mechanism of inhibition of phosphoribosylation of 5-fluorouracil by purines. Biochem Pharmacol 33: 2863–2867, 1984.
36. HOUGHTON JA, and HOUGHTON PJ. Elucidation of pathways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. Eur J Cancer Clin Oncol 19:807–915, 1983.
37. SPEARS CP, SHAHINIAN AH, MORAN RG, ET AL. In vivo kinetics of thymidylate synthetase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinoma. Cancer Res 42:450–456, 1982.
38. MAYBAUM J, ULLMAN B, MANDEL HG, ET AL. Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. Cancer Res 40:4209–4215, 1980.
39. EVANS RM, LASKIN JD, and HAKALA MT. Assessment of growth-limiting events caused by 5-fluorouracil in mouse cells and in human cells. Cancer Res 40:4113–4122, 1980.
40. ARDALAN B, COONEY DA, JAYARAM HN, ET AL. Mechanisms of sensitivity and resistance of murine tumors to 5-fluorouracil. Cancer Res 40:1431–1437, 1980.
41. MCGUIRE JJ, and BERTINO JR. Enzymatic synthesis and function of folylpolyglutamates. Mol Cell Biochem 38:19–48, 1981.
42. PIPER AA, and FOX RM. Biochemical basis for the differential sensitivity of human T- and B-lymphocyte lines to 5-fluorouracil. Cancer Res 42:3753–3760, 1982.
43. STRAW JA, SZAPARY D, and WYNN WT. Pharmacokinetics of diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.
44. NIXON PF, and BERTINO JR. Effective absorption and utilization of oral formyltetrahydrofolate in man. N Engl J Med 286:175–179, 1972.
45. MEHTA BM, GISOLFI AL, HUTCHINSON DL, ET AL. Serum distribution of citrovorum factor and 5-methyltetrahydrofolate following oral and im administration of calcium leucovorin in normal adults. Cancer Treat Rep 62:345–350, 1978.
46. SIROTNACK FM, CHELLO PL, MOCCIO DM, ET AL. Stereospecificity at carbon 6 of formyltetrahydrofolate as a competitive inhibitor of transport and cytotoxicity of methotrexate in vitro. Biochem Pharmacol 28:2993–2997, 1979.

Retinoids as Preventive and Therapeutic Anticancer Agents (Part I)[1,2,3]

S. M. Lippman,[4] J. F. Kessler,[4,5] and F. L. Meyskens, Jr[4,6,*]

Retinoids, the synthetic and natural analogs of vitamin A, frequently block the phenotypic expression of cancer in vitro; they also inhibit growth and induce differentiation in many animal and human malignant cell types. Only recently has it become possible to propose a unifying mechanism of retinoid action, which involves the protein kinase-C cascade system. This system may mediate retinoids' many diverse actions, including their effects on enzyme synthesis, membrane properties, growth factors, binding proteins, genomic and postgenomic expression, the extracellular matrix, and immunologic responses. Ongoing in vitro studies of retinoid structure-activity relationships, effects on oncogene expression, reversal of drug-resistance, and, especially, the protein kinase-C cascade system should help clarify the precise mechanism of their anticancer action. Many in vitro and in vivo assay systems are available for testing the 2000+ synthetic retinoids. These assays indicate specific drug sensitivities, which may help focus future clinical trials. In human cancer prevention, retinoids have been most effective for skin diseases, including actinic keratosis, keratoacanthoma, and basal cell carcinoma; however, nondermatologic premalignancies, such as oral leukoplakia, bronchial metaplasia, laryngeal papillomatosis, cervical dysplasia, myelodysplastic syndromes, and the urinary bladder, also respond to retinoid therapy. Significant therapeutic advances are also occurring with this class of drugs in refractory malignancies, including advanced cutaneous squamous and basal cell cancer, mycosis fungoides, and acute promyelocytic leukemia. Newer third-generation retinoids, such as the highly potent retinoidal benzoic acid derivatives, are demonstrating therapeutic indexes far higher than earlier-generation retinoids. Current in vitro testing is also demonstrating that retinoids have synergistic activity in combination with other agents (eg, biologic modifiers, hormones, and DNA synthesis inhibitors) and treatment modalities (eg, irradiation). Notwithstanding the progress already made with retinoids in human cancer, many in vitro questions remain, and clinical work is just beginning. [Cancer Treat Rep 71:391-405, 1987]

HISTORICAL PERSPECTIVE

"The specific tissue changes which follow the deprivation of fat-soluble vitamin A . . . concerns epithelial tissues. This effect is the substitution of stratified keratinizing epithelium for normal epithelium. . . . The behavior of the replacing epithelium . . . suggests the acquisition of neoplastic properties. . . . We conclude that the deficiency results in loss of specific (chemical) functions of the epitheliums concerned, while the power of growth becomes augmented. . . . *The study of the reverse changes that follow in the rapid amelioration when the rats are restored to an adequate diet has been begun and will be reported later* [italics ours]."—S. Burt Wolbach, MD, and Percy R. Howe, MD, 1925.

With these keen insights and prophetic words began research into the effects of vitamin A and its analogs on cancer—work which would lead to many exciting and promising discoveries in the biology, prevention, and therapy of neoplasia.

The fat-soluble substance essential for growth we now call vitamin A was first recognized in 1909 (1) and named in 1920 (2). In their classic report, Wolbach and Howe (3) demonstrated that a vitamin A-deficient diet led to metaplasia of the gastrointestinal, respiratory, and urogenital epithelia in rats. In 1926, Fujumaki (4) found that frank carcinoma developed in the stomachs of rats deprived of adequate dietary vitamin A. In 1941,

[1] Received Sept 26, 1986; revised Dec 5, 1986; accepted Dec 18, 1986.

[2] Supported in part by Public Health Service grants CA-27502, CA-23874, and CA-23074 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by Hoffmann-La Roche, Inc.

[3] Part II will appear in Cancer Treatment Reports, Vol 71, No. 5, May 1987.

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Abels et al (5) conducted the first studies which associated vitamin A deficiency with human malignancy. Using a mouse prostate organ culture system in 1955, Lasnitzki (6) discovered that premalignant epithelial lesions could be directly suppressed by the addition of retinyl acetate. These studies comprise only a small part of the volumes of information that associate neoplastic disease with vitamin A (7–12).

The recognition that vitamin A deficiency leads to hyperkeratosis of the skin and to squamous metaplasia of mucous membranes directed clinical research toward cutaneous disorders, and substantial clinical benefit has been documented for dermatologic conditions (8). However, the initial excitement was tempered by unacceptable hepatotoxicity in many patients. In the mid-1950s, synthetic vitamin A derivatives were developed in an attempt to produce drugs with an improved therapeutic ratio and now over 2000 analogs have been synthesized (9–12). We now know that retinoids affect the differentiation and proliferation of many cell types, including the following: ectodermal, endodermal, and mesodermal; epithelial, fibroblastic, and mesenchymal; and neoplastic, preneoplastic and nonneoplastic.

In this review, the term "retinoid" is used generically to include the entire set of compounds, both synthetic and natural (excluding carotenoids), that have some or all of the biological activities of vitamin A (retinol). Until now, substantial clinical investigations have been limited to only four retinoids: vitamin A (retinol) and its esters, β-all *trans* retinoic acid (vitamin A acid, or tretinoin), 13-*cis*-retinoic acid (isotretinoin, or Accutane), and an aromatic ethyl ester derivative of retinoic acid (etretinate, or Tegison). Two promising classes of synthetic retinoids now entering clinical trial include the retinamides, eg, *N*-(4-hydroxyphenyl)retinamide (4HPR, or Fenretinide), and the highly potent retinoidal benzoic acid derivatives, eg, TTNPB (fig 1) (10,11).

The basic structure of the retinoid molecule consists of a cyclic end group, a polyene side chain, and a polar end group (fig 1). By modifying any part, different therapeutic ratios can be achieved (11). Chemical manipulation of the polar end group and the polyene side chain produced the first-generation retinoids, which are retinyl esters, amides, aldehydes, ethers, amines, and acids. The most widely studied of these molecules are tretinoin in vitro and isotretinoin in vivo. Second-generation retinoids were developed by altering the cyclic end group. The prototype molecule was etretinate. Cyclization of the polyene side chain has produced the potent third-generation retinoids, such as the retinoidal benzoic acid derivatives (arotinoids). The arotinoid TTNPB (Ro 12-7410) is less toxic and over 1000 times more potent than

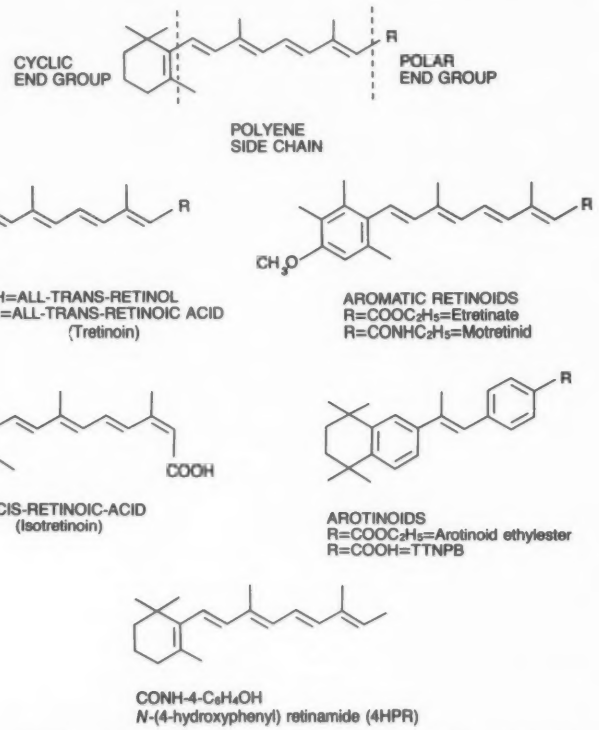

FIGURE 1.—Chemical structures of retinoids. TTNPB = (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid.

tretinoin and retinol in several standard screening tests and is now entering clinical trial. Although the types of side effects among retinoids have been similar (largely differing only in degree), these drugs may exhibit markedly different spectra of activity (7-13).

This review is divided into two main sections. Part I describes the major biological effects and proposed mechanisms of action of retinoids, particularly as they relate to proliferation and differentation processes and their control. Part II details the clinical activity of retinoids as preventive and therapeutic anticancer agents.

BIOLOGICAL PERSPECTIVE

The physiology of vitamin A has been detailed in other reports (13) and will only be summarized here. Vitamin A is obtained from two dietary forms: certain carotenoids, such as β-carotene, which are derived only from plant sources and are not synthesized by animals or man, and the alcohol and aldehyde forms of vitamin A and their esters, which are found chiefly in milk, eggs, and meat. Both forms are also commonly found in over-the-counter pharmaceutical preparations. Although β-carotene can be directly absorbed in small amounts via lymphatic chylomicrons, it is mostly converted to retinol during absorption through the intestinal mucosa, where it and preformed vitamin A (obtained from animal sources) are transported in the plasma by lipoproteins. Vitamin A is then stored in the liver in lipocytes (perisinusoidal stellate, Ito, or fat-storing cells) as retinyl esters, whereas carotenoids are stored in several tissues, primarily in fat and the liver (14). The parenchymal cells store most of the retinyl esters when vitamin A stores are low and have the exclusive role of secreting retinol. There is a dynamic but poorly understood relationship between these cells and the hepatic lipocytes. After storage, the retinyl esters are hydrolyzed into retinol and mobilized from the liver in a highly regulated manner. Retinol is transported in the blood as part of a complex consisting of retinol-binding protein (RBP), transthyretin (formerly called prealbumin), and thyroxine.

Other retinoids have different routes of absorption, storage, and transport. For example, retinoic acid is absorbed primarily through the venous portal route and is rapidly metabolized to intermediates before elimination through both hepatic and renal routes. Retinoic acid's relative absence of hepatotoxicity may be due to its tendency to avoid liver storage.

Naturally occurring vitamin A plays a vital role in vision, growth, reproduction, and epithelial differentiation and proliferation (8,11,15). Metaplastic changes in the epithelium along with increased cell division and nucleic acid synthesis occur in the absence of vitamin A (3,16), and its deficiency enhances the effect of chemical carcinogenesis on epithelial tissues (16,17). Retinoid replacement frequently induces these changes to revert to normal. The regulation of cellular differentiation and proliferation, which will be the focus of this review, is served better by the synthetic acid derivatives than by vitamin A itself.

In the past several years, a large number of investigators have confirmed that retinoids block the phenotypic expression of cancer in humans and animals, regardless of the underlying initiating or promoting factors (18-23). In addition to inhibiting cancer development, retinoids show antiproliferative and differentiation-inducing activity in in vitro cultures of cells from established lines and in human tumor biopsies grown in semisolid medium (19-40). In some cell lines, retinoids have been observed to stimulate the growth and enhance the expression of the transformed phenotype. The complex mechanisms controlling cell proliferation and differentiation and the many factors regulating the transformed phenotype make it difficult to understand clearly what causes different cells to respond divergently to retinoids. Although they can promote cellular transformation, retinoids' anticancer activities far outweigh their potential carcinogenic effect.

In general, the antiproliferative activity of retinoids occurs in a broad range of transformed cell types and is a reversible phenomenon (41,42). It takes effect after 2-3 days of cell exposure to the retinoid and requires about the same amount of time to be reversed, which suggest cell cycle-specific inhibition. Retinoids inhibit proliferation of many malignant cell lines with accumulations of cells in the G_1 phase of the cell cycle, thus slowing their entry into S-phase (43-47). Schroder et al (48) employed flow cytometry to localize the block to the S or G_2 phase in murine Swiss 3T3 cells. In another study, after 24 hours of retinoid exposure, cultured human breast carcinoma cells accumulated in the S-phase. However, after 4-7 days, the cells accumulated in the resting phase (44). Therefore, it appears that in most cells there is a retinoic acid-sensitive G_1 restriction or commitment point. Interestingly, there has been no correlation between retinoid-induced growth inhibition and the cell doubling time (42). Recent evidence suggests that G_1 growth arrest may be regulated separately from differentiation in the HL-60 human promyelocytic cell line (49).

Lotan and Nicolson (42) studied the antiproliferative activity of retinoic acid and retinyl acetate in 31 different cell lines. Although responses in each line differed somewhat from one another, growth was inhibited in $> 60\%$ of the cell lines. The response patterns varied within different cell types, cell lines of the same histologic type, and even in different subclones. This may relate, in part, to differences in cellular retinoid metabolism and stage of transformation (43,50,51). Lotan (52) showed by his studies of murine melanoma S91 cells that a small subset of drug-resistant cells may escape retinoid suppression even within "sensitive" cell lines.

In contrast to the antiproliferative effects, retinoids tend to affect differentiation in a select group of cell types and in a highly specialized, possibly irreversible way (eg, in F9 murine embryonal carcinoma and HL-60

cell lines), and the mechanism(s) by which retinoids alter differentiation may differ from that affecting proliferation (19,53). The evaluation of mutant cell lines should provide further insight into the postulated mechanisms (43,52,54,55). Schindler et al (54), in a murine embryonal carcinoma cell line, used retinoic acid to inhibit growth of a differentiation-defective mutant. They inferred that differentiation and proliferation might be mediated differently. Alternatively, there may be a common mechanism of retinoid action, but the particular response (ie, growth inhibition or differentiation) may depend on the specific cell type. During embryogenic development in vitro, retinol deficiency or excess can selectively inhibit the formation of structures derived from mesenchyme in rat, mouse, hamster, and chick embryos (56). In animals, retinoids have been shown to affect the determination of developing cells by mimicking the action of the polarizing region, thus, for example, altering the development of pattern formation in regenerating chick limb buds (57,58).

In both differentiation and antiproliferation, retinoic acid and its analogs have been, with rare exceptions, consistently more active than retinol, retinyl esters, and retinaldehyde (9–12,30,59). Strickland et al (60) showed that the terminal carboxyl group present in retinoic acid (fig 1), but not in retinol, is essential for the potency of synthetic retinoids as inducers of differentiation in HL-60 and F9 cell lines. Furthermore, the antiproliferative and differentiating effects of retinoids may be biphasic, with high concentrations producing effects completely opposite to those of low concentrations, and the effect of early exposure differing markedly in effect from late exposure, all in the same experimental system.

Moon et al (61) produced some of the strongest evidence for the chemopreventive effects of retinoids in their chemical carcinogenesis studies of the mammary glands of rats. They initiated mammary cancer in rats with 7,12-dimethylbenz[a]-anthracene (DMBA) and found that those rats treated with 2.5 mg of retinyl acetate/day had 52% less cancer than those receiving a placebo diet. Subsequent studies confirmed these results (62,63) and showed that tumor inhibition can be augmented when retinoid treatment is combined with other chemopreventive modalities. Currently the most promising combined treatment involves the interaction between hormonal manipulation and retinoids (44). Either ovariectomy or bromocriptine (a pituitary prolactin inhibitor) plus either retinyl acetate or 4HPR is significantly more effective in cancer prevention than either treatment regimen alone (21,64,65). Similarly, combining 4HPR or retinyl acetate with the antiestrogen tamoxifen is synergistic in preventing rat mammary carcinogenesis.

In vitro studies are also revealing the exciting possibility that retinoids play a critical role in altering drug resistance. Recent studies have shown that retinoic acid selectively inactivates the phosphorylation of certain surface proteins, including P150, which is associated with the reversal of doxorubicin resistance in HL-60 cells (66). Retinoids are also active against certain hormone-resistant transformed cells (67).

MECHANISMS OF ACTION

Despite extensive investigation, the complex mechanism(s) of action which causes the diverse changes modulated by retinoids remains incompletely understood. Although many mechanisms have been proposed, it has only recently become possible to present a unifying hypothesis, which involves the protein kinase-C (PK-C) cascade system, to account for retinoids' varied biological effects. Several recent reviews detail the major cellular and molecular actions of retinoids which have been hypothesized to modulate antiproliferation and differentiation (7,9,11,13,19–22,30,42,51,56,59,60,68). Discussed in order here, the major biological effects include regulation of (*a*) enzyme synthesis, (*b*) membrane function, (*c*) growth factors, (*d*) binding proteins, (*e*) genomic and postgenomic expression, (*f*) extracellular effects, (*g*) immunologic activity, and finally (*h*) the PK-C cascade system.

Retinoids have prominent effects on several enzyme systems, principally the ornithine decarboxylase (ODC)/transglutaminase (TG) and cAMP-dependent and cAMP-independent protein kinases, which appear to be extensively involved in the pattern of cellular response. The biosynthesis of polyamines (spermadine, spermine, putrescine), which have been implicated in the regulation of cellular proliferation and differentiation, relies on the enzyme ODC (69,70). The induction of ODC is a G_1-specific transcriptional event, which is often correlated with tumor promotion and may be obligatory for progression through the cell cycle in normal and neoplastic cells (71,72). In most experimental systems, the inhibition of ODC by retinoic acid and the subsequent depletion of polyamines is directly correlated with growth inhibition, differentiation, and the accumulation of cells in the G_1 phase (43–47,70–78). The best studied in vivo system is that of mouse papillomas induced by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Retinoid inhibition of TPA-induced ODC correlates with the number of papillomas (78). In contrast to DFMO, there is no evidence that retinoids inhibit the activity of the enzyme. Rather they appear to act indirectly by decreasing its synthesis (70). ODC activity itself may be regulated by the actions of intracellular TG. This calcium-dependent enzyme catalyzes the cross-linking of specific membrane-associated proteins, such as fibronectin, and the cross-linking of primary amines to polypeptides (79,80). Retinoids induce a unique soluble TG that may bring about retinoids' antipromotion activity in skin carcinogenesis by antagonizing the phorbol ester-induced increase in epidermal TG (81).

Although many investigators now believe that ODC-induced polyamine biosynthesis is pivotal to the differentiation process, others take exception to this view (70,82–84). For example, Luk et al (82) present contradictory evidence in their study of retinoic acid's effects

on HL-60 cells. Unlike the model of enzyme inhibition presented above, retinoic acid acts to promote differentiation in HL-60 cells not by inhibiting but rather by increasing ODC and polyamines. Luk's group suppressed the ODC increase by adding DFMO and observed that differentiation was unaffected. Therefore, they concluded that ODC activity and consequent biosynthesis of polyamines were not involved in the differentiation process in HL-60 cells.

Luk's results are questioned, however, on the basis of the magnitude of polyamine depletion. Sugiura et al (85) found that nearly complete depletion of spermidine after 60–72 hours of DFMO exposure caused the inhibition of HL-60 cell differentiation induced by retinoic acid. Therefore, although Luk's group did observe ODC inhibition, some investigators believe it was not extensive enough to cause consequent reductions in polyamines to drop below the critical threshold level required to affect differentiation.

Retinoids also affect the calcium cAMP intracellular regulatory system. Fontana et al (86) observed a direct correlation between the degree of retinoic acid-induced differentiation of the HL-60 and human myeloblast cell lines and the activity of cAMP-dependent and phospholipid-sensitive, calcium-dependent protein kinases. Similar correlations in F9 murine teratocarcinoma (87) and B16 and S91 murine melanoma cells (88,89) suggest that these protein kinases and phosphorylation of their substrates are necessary steps in the growth inhibition and differentiation process (90). Retinoid effects were also apparent when agents that elevated cAMP levels acted synergistically with retinoids in inducing differentiation (91). An alternative mechanism of retinoid-mediated protein phosphorylation may involve stimulation of alkaline phosphatase (92).

Retinoids' anticarcinogenic activity may also derive in part from their major effects on other enzyme systems. Retinoic acid has recently been shown in two human cell lines to induce activity of 2′,5′-oligoadenylate synthetase, which is an important enzyme for certain antiviral and antigrowth processes (93). A change in tyrosine phosphorylation is one of the earliest events in chemically induced neoplastic transformation and is involved in the control of certain growth factors. Retinyl acetate's inhibition of tyrosine phosphorylation in 10T 1/2 cells may be an important part of its chemopreventive activity (94). Retinoids also inhibit certain cytochrome P-450 isoenzymes (eg, aryl hydrocarbon hydroxylase) required for activation of cutaneous precarcinogens (eg, DMBA) (95). Their effect of decreasing poly-(ADP-ribose) synthesizing activity is associated with differentiation of mouse teratocarcinoma cells (96). Vitamin A also inhibits TPA-induced epidermal oxidized glutathione reductase activity in mice (97), induces de novo synthesis of sialyltransferase and galactosyltransferase in F9 cells (98), and regulates acetylcholinesterase in human LA-N-5 neuroblastoma cell lines (99). Retinoids also affect other enzymes which may play a role in their anticarcinogenic activity (100–102).

Retinoids influence the synthesis and distribution of membrane glycoproteins, glycolipids, proteoglycans, and glycosaminoglycans (30,103–108). Retinoic acid inhibits the mobility of surface anions of sarcoma and melanoma cells (109). The synthesis, distribution, and binding of cell-associated fibronectin, a large surface glycoprotein which is quantitatively reduced in transformed cells, is altered by retinoid exposure (84,105,109–111). Increased release of fibronectin may be a required event for tumor promotion and transformation. Retinoic acid antagonizes TPA-induced fibronectin release in promotable clones of human lung fibroblasts and a JB6 mouse epidermal cell line (111–113). Retinoids prevent redistribution of cell surface, negatively charged sialoglycoconjugates and glycosaminoglycans in two human sarcoma cell lines, which may be due to retinoid-stimulated sialyltransferase activity and glycosylation of specific membrane proteins (105,112,114). Retinoids also modulate the number of specific surface receptors, such as insulin-binding sites on human histiocytic lymphoma (U-937) cell lines and specific 1,25-dihydroxyvitamin D_3 receptors in osteosarcoma cells, which are associated with the induction of differentiation (115–117).

Cell membrane glycoconjugate alterations affect cell-to-cell and cell-to-substratum interactions, including cell adhesion, contact inhibition, migration, differentiation, growth, and transformation (118). All these interactions may affect tumor behavior. Indeed, inhibition of tumor growth of S91 murine melanoma cells sequentially follows the enhancement by retinoids of glycosylation of a specific membrane glycoprotein (gp 160) of melanoma cells, which suggests a causal relationship (52,119). Retinoic acid inhibits the capacity of nonneoplastic keratinocytes and the transformed SQ cc/yl cell line for stratification and terminal differentiation which leads to premature desquamation (120). This effect is accompanied by specific alterations in the composition of membrane-associated glycosaminoglycans and in the expression of keratin intermediate filaments (120,121). However, retinoic acid regulates the production of specific glycoconjugates in HL-60 cells independently of its effects on cell differentiation (122). One attractive proposal is that retinoids participate indirectly as a cofactor in sugar transfer reactions via the formation of a retinyl phosphate mannose intermediate (56,123–125). However, evidence is limited, and at best this is a minor mechanism since the arotinoids, which are among the most potent retinoids, would not participate structurally in these reactions (126). Furthermore, in melanoma, HL-60, and other cell lines, retinoic acid modifies membrane glycoprotein sialylation by inducing the enzymes sialyltransferase, glycosyltransferase, and fucosyltransferase rather than by working as a cofactor (127).

In addition to these specific biochemical effects, retinoids stimulate the synthesis of gap junctions and enhance intercellular communication (118,128). Retinoic acid can restore density-dependent contact inhibition of growth to transformed mouse L929 cells. They also change membrane microviscosity and electrophysiologic

properties and at high concentrations exert a direct detergent-like action on cell membranes, which may produce both cytotoxic and undesirable toxic responses (129). Anchorage-independent growth is highly correlated with neoplastic growth in vivo and retinoids inhibit this property in a wide variety of transformed cells (130). These diverse effects on cellular membranes, along with the effects of certain growth factors, may explain why cells grown under anchorage-independent conditions are more sensitive to retinoids than anchorage-dependent-grown cells (68,131).

Retinoids modulate the effects of many mitogens and transforming growth factors which affect the cell membrane, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), colony-stimulating factor (CSF), sarcoma growth factor derived from the Moloney murine sarcoma virus-transformed 3T3 cells, other tumor promoters (eg, teleocidin), and hormones (eg, insulin) (132–144). EGF, one of the best-studied and most important growth factors, regulates growth and differentiation of epithelial and mesenchymal cells and plays a role in embryogenesis and neoplastic transformation (137–139). EGF interacts with many enzyme systems. Most notably it increases the activity of ODC and the levels of putresine in vivo and in vitro (70,139,140). EGF is integrally involved in the PK-C cascade (139,141). It also increases diacylglycerol (DG) production which activates PK-C. The activated PK-C then phosphorylates the EGF receptor which inhibits binding of EGF. This feedback mechanism controls EGF activity. Loss of EGF requirement for growth in vitro has been associated with malignant transformation (138). Jetten (132) showed in several cell lines that retinoids enhance EGF binding by producing an increase in EGF receptor sites without affecting receptor activity. These increases correlated with growth inhibition in the mouse fibroblast 3T6 cell line and with differentiation in the embryonal carcinoma cell lines. Although the way retinoic acid induces the increase in the EGF receptor number is not known (144), a recent study of rat tracheal epithelial 2C5 cells by Jetten and Shirley (142) suggests that this effect is not mediated through PK-C. In studies of CSF and hematopoietic progenitors, Tobler et al (143) found that retinoids either recruit earlier progenitors to the GM-CFC pool or enhance the sensitivity of GM-CFC to the action of CSF. The latter effect is possibly achieved by increasing the number or affinity of the CSF surface receptors. The precise influence of these mitogens on carcinogenesis, differentiation, and proliferation remains to be defined.

Initially, retinoids were thought to alter gene expression in a way very similar to the steroid model (25), but work in the last few years has not verified this concept. There is evidence that the retinoid combines with either a cellular retinol-binding protein (cRBP) or cellular retinoic acid-binding protein (cRABP) once it is delivered to the appropriate target tissue. Both retinol and its derivatives and retinoic acid and its synthetic analogs show high specificity for their respective intracellular binding proteins (13). These different binding proteins are independently controlled, which indicates that retinol and retinoic acid affect the regulation of growth and differentiation in different ways. What happens next has eluded the understanding of investigators (51,56,59,68). There are an estimated 100,000–300,000 binding sites per nucleus for cRBP and slightly fewer for cRABP (145). However, the binding is to nuclear membranes, not to DNA (51). Furthermore, in contrast to the steroid receptor protein, the specific DNA sequences whose transcriptions are regulated by retinoid-binding proteins have not been identified.

Possibly some malignancies express higher numbers of the cellular binding proteins, which may enhance their susceptibility to the differentiating or antiproliferating effects of retinoids. The generally higher concentrations of cRBP in tumor compared to normal tissue have been demonstrated in many human and experimental tumors and in many cultured cell lines (146). Retinoid responsiveness of mouse F9 and tracheal epithelial cells (eg, inhibition of the formation of cross-linked envelopes and reversal of keratinization) correlates with the presence of cRABP (37,51,126,147). Furthermore, recent data suggest that cRABP mediates the increase in cAMP-dependent protein kinases which are associated with retinoic acid-induced differentiation of embryonal carcinoma cell lines (148). In contrast, some retinoid responsive cell lines (eg, HL-60) do not express cRABP(41,149). In addition, human embryonal carcinoma cells and other human teratocarcinoma-derived cell lines do not differentiate in culture in response to retinoic acid, nevertheless they express high levels of cRABP (150). Also, Lotan (29) discovered that some melanoma cell lines which were resistant to the effects of retinoids contained levels of binding proteins comparable to or higher than levels in sensitive cell lines. These data suggest that retinoid-binding proteins alone may not confer antiproliferative properties.

The hypothesis compatible with the widest range of in vitro and in vivo data is that retinoids modify genomic expression. Green and co-workers (151,152) showed that retinoids mediated the suppression of the synthesis of a 67-kDa keratin at the level of mRNA synthesis and were involved in the transcriptional activation of 40-kDa and 52-kDa keratins in human epidermal keratinocytes. Similar observations associated with retinoid-induced differentiation have been reported in other systems such as murine teratocarcinoma stem cells and HL-60 promyelocytic cells (153–156). Recently, Wang et al (155) used hybridization selection and in vitro translation to identify several independent retinoid-inducible gene sequences in F9 cells, demonstrating for the first time that retinoids regulate the transcription of specific genes.

Retinoids may also antagonize the action of oncogenes or their products, which are normally genetically repressed polypeptides that change the regulated growth pattern of normal cells to a more malignant pattern. The c-myc oncogene plays an important role in the dif-

ferentiation process of the HL-60 cell line. Westin et al (157) provided evidence that retinoic acid modifies c-myc gene transcription. Expression of the myc gene was turned off by retinoic acid. This lack of expression was not due to resting differentiated cells, and suppression of c-myc was unassociated with suppression of the c-abl cellular oncogene. Recently it has been shown that retinoic acid-induced c-myc transcriptional down regulation in HL-60 cells occurs at the level of elongation, not initiation (158). The position of the elongation was mapped to the boundary between exon 1 and intron 1. In ML-1 cells, c-myb expression declined early after retinoic acid treatment, which was also associated with the loss of cell proliferation (159).

N-myc, a DNA sequence which shares limited homology with c-myc, is amplified in neuroblastoma cell lines. A recent study showed that during retinoic acid-induced differentiation of SMS-KCNR neuroblastoma cells (160), a decreased level of N-myc expression preceded cell-cycle changes and morphologic differentiation. In addition, N-myc mRNA content was decreased in the LA-N-5 neuroblastoma cell line following retinoic acid-induced morphologic differentiation and growth inhibition in both monolayer and soft agar culture (161). In contrast to the suppression of c-myc expression in the HL-60 cell line, analysis of the level of v-onc mRNA revealed that inhibition by retinoic acid of retroviral transformation of normal rat kidney and NIH/3T3 cells was not due to a decrease in transcription of the v-onc genes (162). These and other results suggest that the mediation of transformation at the molecular level by retinoic acid may be heterogeneous, depending on the cell type and/or the transforming agent (59,60,144,163,164).

Jetten et al (130) provided recent evidence that the activation of specific oncogenes in transformed DES-4 Syrian hamster embryo cells can influence their response in vitro to retinoids. Reductions in saturation density, anchorage-independent growth, and TPA-induced ODC activity indicated that retinoic acid inhibited the activities of the transformed phenotype in src-453 cells in which the v-src oncogene is activated. In contrast, retinoic acid stimulated the activity of the transformed phenotype and increased ODC activity in $rasC_2$ cells, in which the v-Ha-ras oncogene is activated. Jetten further demonstrated that v-src- and v-Ha-ras-transfected 10W cells (also derived from Syrian hamster embryo cells) responded differently to retinoids than the similarly transfected DES-4 cells. This proves that oncogenes alone do not determine the cellular response to retinoids. Although the mechanism by which retinoic acid interferes with the expression of oncogene products is not known, Jetten speculates that retinoic acid may influence v-src and v-Ha-ras activities by playing a critical role in the synthesis of and response to endogenous growth factors, which are regulated by these oncogenes.

Investigation of the susceptibility of F9 murine teratocarcinoma stem cells to transformation by papovavirus (SV40) has suggested that retinoids might control protein synthesis by posttranscriptional control of RNA processing (165). The posttranscriptional regulation of mRNAs encoding other proteins such as alpha-tubulin in F9 cells has been reported (166). Wolf et al (125) provided evidence for the posttranscriptional control by retinoids of glycoprotein synthesis through the formation of glycosyl phosphoryl retinoid intermediates. Retinoids have also been shown to expand the cellular ATP pools in Swiss mouse 3T3 cells, which led to inhibition of DNA replication (167).

Retinoids also have major effects which are expressed extracellularly, including altering the synthesis of plasminogen activator, collagenase, and certain prostaglandins. Primitive ectodermal cells produce plasminogen activator, which is thought to be a specific marker for differentiation in early embryogenesis and for retinoid-dependent differentiation of F9 teratocarcinoma stem cells (35,51). In other cultured human cells and tumor cell lines, however, retinoids have produced variable effects on plasminogen activator synthesis (168). Retinoids inhibit synovial and skin collagenase synthesis in a process that is synergistic with steroids (169). This mechanism may involve inhibition of the synthesis of collagenase mRNA or, alternatively, may involve the augmentation of an autoregulator protein. The beneficial activity retinoids have shown in proliferative disorders such as experimental arthritis may be based on this inhibition of collagenase synthesis (170). These results have clinical implications for the treatment of many other disorders characterized by excessive synovial and skin collagenase production, such as psoriatic and rheumatoid arthritis and epidermolysis bullosa dystrophica. Skin carcinogenesis is enhanced by certain prostaglandins and inhibited by retinoids and/or prostaglandin inhibitors. An important mechanism of retinoid action may thus be the inhibition of prostaglandin synthesis or antagonism of its effects (8,40,171).

Retinoids have diverse effects on both the resting and activated immune system (104,172–174). In mice, low retinoid doses stimulate and high doses inhibit certain immune functions. Vitamin A potentiates antibody formation in mice which have been injected sc with a normally nonimmunogenic bovine protein or which have been sensitized to sheep rbcs. Retinol and retinoic acid increase the phagocytic and tumoricidal activity of macrophages (174,175) and activate superoxide production of polymorphonuclear leukocytes (176,177).

Extensive studies have shown that retinoids augment cell-mediated cytotoxicity in vivo and in vitro in certain syngeneic and allogeneic tumor model systems (178,179). They also inhibit antibody-dependent cellular cytotoxicity, possibly by modulating membrane receptors (180). Retinoids may have a broad effect on interleukin (IL)-2-dependent immune functions (173). They stimulate production of IL-2 by T-helper cells, which results in T-killer cell proliferation. Mice injected with retinoids display an enhanced capacity to produce IL-2 on stimulation of their splenocytes in mixed lymphocyte reaction. Independently of IL-2-dependent proliferation,

retinoic acid also directly augments mature thymocyte (OKT3+ OKT6−) proliferation to PHA activation (181). However, some retinoids appear to be directly toxic to lymphocytes in vitro and may induce decreased formation of E-rosettes. Vitamin A also enhances cytotoxic T-cell-mediated tumor rejection in mice (182).

Eccles (172) studied the effects of the aromatic retinoic acid analog (Ro 10-0359) on in vivo growth and metastases of 14 transplantable syngeneic tumors (183). In all cases, the local tumor-inhibitory effect was T-cell dependent, and the accessory cell function of macrophages was important in preventing metastases. If the animals were rendered incapable of evoking a T-cell response, retinoids were unable to influence tumor growth. For example, this phenomenon was abolished in congenital athymic nude mice and in mice that received thymectomy plus irradiation along with cytarabine (Ara-C) priming or with cyclosporine A, which interferes with the IL-2 response (172). Retinoic acid also plays a role in natural killer (NK) cell induction. Split-dose irradiated mice that lose their NK activity can be protected from developing leukemia either by reconstitution with cloned NK cells or by retinoid injection, which suggests that retinoids may stimulate recovery of NK activity (173). Alternatively, retinoic acid may have a direct effect on blocking the phenotypic expression of leukemia.

Other promising examples of retinoid action include enhanced rejection of skin homografts, stimulation of thymocyte proliferative responses, and promotion of delayed cutaneous hypersensitivity. These properties may be responsible, in part, for the tumor responses seen in vivo (179,184). For example, mice treated with retinyl palmitate required less irradiation to eliminate immunogenic tumors than did untreated mice. Also, retinyl palmitate apparently potentiates the antitumor effect of the immune-enhancer *Mycobacterium bovis* (strain BCG). Despite these many effects of retinoids on the immune system, the retinoid target cell has not yet been convincingly identified.

A unifying hypothesis for retinoids' mechanism of action, which may underlie the significant actions detailed above, involves the PK-C/phosphoinositol cascade system (141,185). PK-C, the phorbol ester receptor, is a Ca^{2+}-activated, phospholipid-dependent enzyme, which is a major cell surface signal transduction pathway, and plays a critical role in the carcinogenic process (141). Phorbol esters, such as TPA, and endogenous ligands, such as DGs, activate PK-C directly in vitro and in vivo. Recently, PK-C has been implicated in ODC induction and EGF receptor down regulation (142). Membrane-bound activated PK-C may transmit a signal to the nucleus to increase gene (eg, the ODC gene) transcription. PK-C may regulate gene expression by phosphorylating proteins such as histones and may alter oncogene (eg, c-myc) expression. In contrast, PK-C's control of cell response to EGF is accomplished directly by phosphorylating the EGF receptor and possibly by inhibiting internalization of EGF, which are nontranscriptional events (142).

The theory that retinoids modulate PK-C activity is supported by recent research by Cope and others (185). The effects of retinoids on PK-C are complex and vary with the experimental system employed (186). Although the precise way retinoids interact with PK-C at the molecular level is not known, they do not directly affect phorbol ester binding to PK-C (132,185). Cope's studies of mouse brain PK-C propose an exciting idea of how this interaction may work. They have shown that whereas unbound retinoid-binding proteins (Apo-cRABP and Apo-cRBP) are substrates for PK-C, the holo-forms (eg, retinoic acid-cRABP) inhibit PK-C activity. The relative effectiveness of different retinoids in inhibiting PK-C activity in Cope's system correlated with each retinoid's ability to inhibit ODC and induce differentiation in F9 cells. The regulation of PK-C alters phosphorylation of the retinoid-binding proteins (RBPs) as well as other specific cytosolic and membrane substrates, which could explain the myriad of reported actions of retinoic acid and provide a unifying concept of its anticancer activity. Furthermore, interaction with the PK-C cascade system may explain the synergistic anticarcinogenic activity of retinoids with other agents, such as antiestrogens (21,62–65,187).

Although many investigators now believe that PK-C occupies a central role in retinoid action, the precise details of Cope's model are challenged by recent data from Jetten and others. In rat tracheal epithelial cells, Jetten (142) showed that retinoic acid does not inhibit ODC by blocking PK-C activation but acts to inhibit ODC induction at a stage following PK-C activation. Furthermore, in the same cell system they showed that retinoic acid's effect on EGF binding is not mediated through PK-C as is ODC inhibition. The role of cRABP in Cope's model is questioned by Lockyer's data showing that the retinoic acid-induced increase in EGF binding in 3T3 cells is not related to cRABP (140). Furthermore, although retinoid responsiveness in most cells correlates with RBP number and affinity, some responsive cell lines (eg, HL-60) do not have RBPs which are detectable by conventional ligand binding methods. Cope, however, recently documented the presence of RBPs in mouse brain plasma membrane, which suggests that RBPs could be present in retinoid-responsive cells that have no apparent cytosolic RBPs (185).

PRECLINICAL SCREENING

Details of the biological assays of retinoids and their structure-activity relationships have been reviewed elsewhere and will only be discussed briefly here (11,188–190). The original biologic assay used to discover retinol and retinyl esters was developed 75 years ago and measures the ability to support growth in vitamin A-deficient rats (191). There are now numerous in vitro and in vivo assays available for testing the > 2000 synthetic retinoid derivatives.

The most widely employed methods for retinoid screening have been the mouse papilloma assay of Bollag and

the hamster tracheal organ culture system popularized by Sporn. First described in 1947, the mouse papilloma assay has undergone many subsequent variations, including Bollag's (11,192,193). All of these assays exploit three well-studied retinoid effects on experimental skin carcinogenesis, including: (*a*) inhibition of the primary development of mouse skin papilloma initiated by DMBA and promoted by TPA; (*b*) regression of the papillomas once they have developed; and (*c*) suppression of the neoplastic progression of benign skin papilloma to frank carcinoma. Bollag's standard assay measures the ability of retinoids to cause regression of established carcinogen-induced papillomas. The initiator DMBA is applied twice at 14-day intervals to shaved dorsal skin of mice. Three weeks later, the promoter (croton oil in acetone) is applied twice a week for 3–8 months until multiple papillomas develop. At this point, intraperitoneal, or oral, retinoid is begun on a daily basis. The sum of the papilloma diameters present on Day 14 of retinoid therapy is compared to the sum of diameters that existed when therapy began. Using this assay, Bollag has defined the therapeutic index in terms of the ratio between the lowest daily ip dose causing a defined degree of hypervitaminosis A in a 14-day study and the ip dose causing 50% regression of all papillomas.

The hamster tracheal organ culture assay measures the ability of retinoids to reverse the keratinization of the squamous metaplasia expressed by vitamin A-deficient hamster trachea (16,194). Tracheas in the early stage of vitamin A deficiency are placed in organ culture without retinoid for 3 days. Within this time, significant squamous metaplasia develops, and the trachea are divided into a control culture with DMSO and a second culture of DMSO plus retinoid. After 10 days of incubation, the tracheas are harvested and fixed. They are scored microscopically for the presence of keratin and keratohyaline granules. Retinoids are considered inactive if both of these histologic changes are present or active if neither is present.

Although these screens have been predictive of efficacy for benign and malignant epithelial disorders, additional systems are needed for screening retinoids' effects on other tissue types and disease states. It would be unfortunate if we failed to identify retinoids that could be effective in nonepithelial diseases simply because we relied exclusively on one or two epithelial-oriented screens. Therefore, many other experimental systems have been explored for retinoid activity. These include in vitro cell culture, in vitro organ culture, and in vivo approaches.

In vitro assay systems using cell culture include: increase of nucleic acid synthesis in epidermal cell cultures, inhibition of growth of murine melanoma B16 and S91 cells, regulation of differentiation and specific keratin gene activity, suppression of neoplastic transformation of mouse fibroblasts, and enhancement of adhesion in transformed mouse fibroblasts (48,84,88,89, 152,190). The murine F9 embryonal carcinoma and human HL-60 promyelocytic cell lines are currently the most widely employed systems both for screening purposes and to investigate the mechanisms of retinoid action (24,35,46,49,51,54,55,60,79,83,86,87,98,108,122,124, 126,143,147,149,153–155,157,158,165,166). In addition to these two systems, many transformed human cell lines are available (eg, neuroblastoma, retinoblastoma, squamous cell carcinoma, small and non-small cell lung carcinoma, breast, teratoma, teratocarcinoma, ovarian, lymphoma, and leukemic), and new assays are continually being developed to screen retinoids both alone and in combination with hormones, other biologic modifiers, and DNA synthesis inhibitors (28,29,32–34,36,47,50,99, 105,115,159–161,195–201). For example, investigators now are exploring the use of estrogen receptor-positive and receptor-negative human breast cell lines in studying the effects of retinoids in combination with antiestrogens (202–204). Assessment of the effect of retinoids on human tumor colony-forming cells may also be helpful in the initial screening for therapeutic activity (26,31,33). The predictive capacity of in vitro cell-free systems, such as measurements of binding affinity to cRABP, have not been adequately explored (37,38).

The in vitro screening methods using organ culture include the following: suppression of keratinization and induction of mucous metaplasia in embryonic chick skin, inhibition of the induction of ODC and terminal differentiation by calcium and phorbol esters in mouse and human epidermis, suppression or reversal of the effects of carcinogens on mouse prostate and mammary glands, embryonic mouse epidermis limb-bud organ culture, toxic effects of retinoids on bone and cartilage in organ culture, and reversal of keratinization in vitamin A-deficient rabbit or hamster tracheas (169,205–211).

Many in vivo methods are also used, which measure retinoid effects on growth support, growth promotion, carcinogenesis, and differentiation in several model systems (205,212–217). Although the study of transplantable tumors has led to improved understanding of retinoic acid-mediated immune effects, the majority of allogeneic and syngeneic transplantable tumors used to screen for retinoic acid's antitumor activity have been more difficult to interpret than the in vivo (eg, Bollag model) and in vitro (eg, cell culture) methods discussed above. These tumor studies have also produced variable and often negative results (172,178,179,183,217). However, the current availability of many animal and human transplantable homogeneous transformed cell lines may provide useful screens for retinoid antitumor activity. Recently, Levine (218) described a novel way to study retinoid effects on melanoma. In his in vivo model system, S91 murine melanoma cells were inoculated into upper dermal blister cavities created by negative suction pressure in the skin of DBA/2 mice. The resulting tumor nodules resembled cutaneous metastases of melanoma because of their superficial dermal location. Topical applications of tretinoin at varying concentrations in DMSO, begun 1 day after inoculation and continuing for 28 days, prevented development of visible or palpable tumors and decreased incorporation of (^{14}C)-thiouracil, a measure of melanoma growth, in a dose-dependent manner. This model may be useful in study-

ing the mediation of host-tumor responses by retinoids.

The screening of new derivatives for toxicity will be as critical to the future development of retinoids as the demonstration of efficacy. A major advance would be to find new retinoids equal in efficacy to existing agents but significantly less toxic at therapeutic doses. In fact, some of the recently synthesized retinoids lack the systemic toxic effects such as alterations in liver function tests and serum lipids, teratogenicity, and bone toxicity (219,220). Combining retinoids at lower doses with other agents, including cytotoxic drugs, hormones and/or biological modifiers, provides another strategy for enhancing their use.

STRUCTURE-FUNCTION RELATIONSHIPS

Definition of the structure-function relationships of retinoids in the various in vivo and in vitro assay systems has facilitated the identification of specific skeletal components of the retinoid molecules which may mediate antiproliferation and/or differentiation for a specific malignant cell type (11,60,143,188–190,221–224). In addition to providing direction to clinical study, analysis of these structure-activity relationships should significantly aid our understanding of the mechanism of retinoid action. Strickland et al (60) have determined the importance of the retinoid skeleton's terminal carboxyl group for differentiation of HL-60 and F9 cell lines. Recently, Tobler et al (143) extended this analysis in both normal and leukemic HL-60 and KG-1 human cell lines exposed to ten structurally different retinoids. They showed that retinoic acid derivatives with a carboxyl group were significantly more active for both stimulation of normal hematopoiesis as well as inhibition of clonal growth of leukemic cells than were retinoids with a derivatized carboxyl moiety. This observation suggests that an important common structural requirement for the modulation of either normal or abnormal hematopoiesis may exist. Furthermore, although the terminal carboxyl group was necessary for binding retinoid to cRABP, it is not certain that cRABP mediates differentiation in leukemic cells since it is not detectable in KG-1, HL-60, and cells harvested from the bone marrow of patients with acute nonlymphocytic leukemia (220).

Yen et al (223) recently showed that isomerization of the alkyl chain could uncouple the capacity of HL-60 cells to undergo G1/0 growth arrest from the property that affects myeloid differentiation. Their results indicated that *cis-trans* isomerization of the alkyl chain affects the capability of retinoic acid to drive later-than-precommitment cellular events which lead to differentiation. Another recent study by Fabian and Shvartzmayer (224) showed that TTNPB is superior to 13-*cis*-retinoic acid in stimulating normal BFU-E without affecting their self-renewal capacity. This result suggests that TTNPB increases the percentage of progenitors responding to CSF. The chemical specificity of these effects is demonstrated dramatically by the abolition of biologic activity resulting from moving the carboxyl group of TTNPB from the para to the meta position on the benzene ring (225).

The specificity that different synthetic analogs have for certain malignant cell types is exemplified by the retinamide 4HPR. This analog has a high therapeutic index against chemically induced tumors of the rat mammary gland and urinary bladder and hamster trachea; however, it has limited activity in hematopoietic cells such as the HL-60, KG-1, and normal human GM-CFC cell systems (65,143). Further evaluation of the structure-function relationships of the different retinoids in the various in vivo and in vitro assay systems will undoubtedly lead to more focused clinical trials.

REFERENCES

1. STEPP W. Versuche uber Futterung mit lipoidfreir nahrung. Biochem Z 22:452-460, 1909.
2. DRUMMOND JC. The nomenclature of the so-called accessory food factors (vitamins). Biochem J 14:660, 1920.
3. WOLBACH SB, and HOWE PR. Tissue changes following deprivation of fat soluble A vitamin. J Exp Med 42:753-777, 1925.
4. FUJUMAKI Y. Formation of gastric carcinoma in albino rats fed on deficient diets. J Cancer Res 10:469-477, 1926.
5. ABELS JC, GORHAM AT, PACK GT, ET AL. Metabolic studies in patients with cancer of the gastrointestinal tract. I. Plasma vitamin A levels in patients with malignant neoplastic disease, particularly of the gastrointestinal tract. J Clin Invest 20:749-764, 1941.
6. LASNITZKI I. The influence of A hypervitaminosis on the effect of 20-methylcholanthrene on mouse prostate glands grown in vitro. Br J Cancer 9:434-441, 1955.
7. KUMMET T, and MEYSKENS FL, JR. Vitamin A: a potential inhibitor of human cancer. Semin Oncol 10:281-289, 1983.
8. PECK GL. Retinoids in clinical dermatology. *In* Progress in Diseases of the Skin (Fleischmajer R, ed). San Francisco, Grune and Stratton, Inc, 1981, vol 1, pp 227-269.
9. BOLLAG W. Retinoids and cancer. Cancer Chemother Pharmacol 3:207-215, 1979.
10. LOELIGER P, BOLLAG W, and MAYER H. Arotinoids, a new class of highly active retinoids. Eur J Med Chem Ther 15:9-15, 1980.
11. BOLLAG W. Vitamin A and retinoids: from nutrition to pharmacotherapy in dermatology and oncology. Lancet 1:860-863, 1983.
12. CUNNINGHAM WJ, and EHMANN CW. Clinical aspects of the retinoids. Semin Dermatol 2:145-160, 1983.
13. WOLF G. Multiple functions of vitamin A. Physiol Rev 64:873-937, 1984.
14. DELUCA LM, and CREEK KE. Vitamin A and the liver. *In* Progress in Liver Disease (Popper H, and Schaffner F, eds). San Francisco, Grune and Stratton, Inc, 1986, vol xxxv, pp 81-98.
15. MOORE T. Effect of vitamin A deficiency in animals: pharmacology and toxicology of vitamin A. *In* The Vitamins, 2nd ed (Sebrell WH, and Harris RS, eds). New York, Academic Press, 1967, pp 245-294.
16. HARRIS CC, KAUFMAN DG, SPORN MB, ET AL. Histogenesis of squamous metaplasia and squamous cell carcinoma of the respiratory epithelium in an animal model. Cancer Chemother Rep Part 3, vol 4(2):43-54, 1973.
17. NETTESHEIM P, SNYDER C, and KIM JCS. Vitamin A and the susceptibility of respiratory tract tissues to carcinogenic insult. Environ Health Perspect 29:89-93, 1979.
18. BOUTWELL RK. Biochemical mechanism of tumor promotion. *In* Carcinogenesis, Mechanisms of Tumor Promotion and Carcinogenesis (Slaga TJ, Sivak A, and Boutwell RK, eds). New York, Raven Press, 1978, vol 2, pp 49-58.
19. Discussion: Mammary differentiation and bladder carcinogenesis.

In Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 182–190.
20. MEYSKENS FL, JR. Modulation of abnormal growth by retinoids: a clinical perspective of the biological phenomenon. Life Sci 28: 2323–2327, 1981.
21. MOON RC, MCCORMICK DL, and MEHTA RG. Inhibition of carcinogenesis by retinoids. Cancer Res 43:2469–2475, 1983.
22. SPORN MB, DUNLAP NM, NEWTON DL, ET AL. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed Proc 35:1332–1338, 1976.
23. MEYSKENS FL, JR. Prevention and treatment of cancer with vitamin A and the retinoids. *In* Vitamins, Nutrition and Cancer (Prasad K, ed). Basel, Karger, 1984, p 266.
24. BREITMAN TR, SELONICK SE, and COLLINS SJ. Induction of differentiation of the human promyelocytic cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 77:2936–2940, 1980.
25. CHYTIL F, and ONG DE. Mediation of retinoic acid induced growth and anti-tumor activity. Nature 260:49–51, 1976.
26. COWAN JD, VON HOFF DD, DINESMAN A, ET AL. Use of a human tumor cloning system to screen retinoids for antineoplastic activity. Cancer 51:92–96, 1983.
27. ETTLIN R, GALLI B, and KISTLER A. Histological changes during regression induced by retinoic acid in a transplantable rat chondrosarcoma. Virchows Arch 396:1–8, 1982.
28. LACROIX A, and LIPPMAN ME. Binding of retinoids to human breast cancer cell lines and their effects on cell growth. J Clin Invest 65:586–591, 1980.
29. LOTAN R. Different susceptibilities of human melanoma and breast carcinoma cell lines to retinoic acid-induced growth inhibition. Cancer Res 39:1014–1019, 1979.
30. LOTAN R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochim Biophys Acta 605:33–91, 1980.
31. MEYSKENS FL, JR, ALBERTS DS, and SALMON SE. Effect of 13-*cis*-retinoic acid and 4-hydroxyphenyl-all-*trans*-retinamide on human tumor colony formation in soft agar. Int J Cancer 32:295–299, 1983.
32. MEYSKENS FL, JR, and FULLER BB. Characterization of the effects of different retinoids on the growth and differentiation of a human melanoma cell line and selected subclones. Cancer Res 40:2194–2196, 1980.
33. MEYSKENS FL, JR, and SALMON SE. Inhibition of human melanoma colony formation by retinoids. Cancer Res 39:4055–4057, 1979.
34. SIDELL N. Retinoic acid induced inhibition and morphological differentiation of human neuroblastoma cells in vitro. JNCI 68:589–593, 1982.
35. STRICKLAND S, and MAHDAVI V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell 15:393–403, 1978.
36. THEIN R, and LOTAN R. Sensitivity of cultured human osteosarcoma and chondrosarcoma cells to retinoic acid. Cancer Res 42: 4771–4775, 1982.
37. TROWN PW, PALLERON AV, and BOHOSLAWRE O. Relationship between binding affinities to cellular retinoic acid-binding protein and in vivo and in vitro properties for 18 retinoids. Cancer Res 40:212–220, 1980.
38. WIGGERT B, RUSSEL P, LEWIS M, ET AL. Differential binding of soluble nuclear receptors and effects on cell viability of retinol and retinoic acid in cultured retinoblastoma cells. Biochem Biophys Res Commun 79:218, 1977.
39. SAIGA T, WIERENGA DE, LEVENBOOK I, ET AL. Comparison of the colony forming ability and invasive potential of six primate cell lines treated with retinoic acid. Invest New Drugs 4:25–29, 1986.
40. BREGMAN MD, PETERS E, SANDER D, ET AL. Dexamethasone, prostaglandin A and retinoic acid modulation of murine and human melanoma cells grown in soft agar. JNCI 71:927–932, 1983.
41. DOUER D, and KOEFFLER HP. Inhibition of the clonal growth of human myeloid leukemia cells. J Clin Invest 69:277–283, 1982.
42. LOTAN R, and NICOLSON GL. Inhibitory effects of retinoic acid or retinyl acetate on the growth of untransformed, transformed and tumor cells in vitro. J Natl Cancer Inst 59:1717–1722, 1977.
43. GALLAGHER RE, CHANG C-S, and SCHWARTZ EL. (Abstr) Metabolism of retinoic acid (RA) by wild-type (wt) and RA-resistant HL-60 cells: marked effect of serum. Blood 68(suppl 1):188a, 1986.
44. MARTH C, MAYER I, and DAXENBICHLER G. Effect of retinoic acid and 4-hydroxytamoxifen on human breast cancer cell lines. Biochem Pharmacol 33:2217–2221, 1984.
45. TRAGANOS F, HIGGINS PJ, BUETI C, ET AL. Effects of retinoic acid versus dimethylsulfoxide on Friend erythroleukemia cell growth. II. Induction of quiescent nonproliferating cells. JNCI 73:205–218, 1984.
46. YEN A, REESE SL, and ALBRIGHT KL. Control of cell differentiation during proliferation. II. Myeloid differentiation and cell cycle arrest of HL-60 promyelocytes preceded by nuclear structural changes. Leuk Res 9:51–71, 1985.
47. CRAIG RW, FRANKFURT OS, SAKAGAMI H, ET AL. Macromolecular and cell cycle effects of different classes of agents inducing the maturation of human myeloblastic leukemia (ML-1) cells. Cancer Res 44:2421–2429, 1984.
48. SCHRODER EW, RAPAPORT E, KABCENELL AK, ET AL. Growth inhibitory and stimulating effects of retinoic acid on murine 3T3 cells. Proc Natl Acad Sci USA 79:1549–1552, 1982.
49. VITALE C, RAKOWKI I, and RAZA A. (Abstr) Number of DNA replication cycles completed by HL-60 cells during differentiation induced by 13 *cis*-retinoic acid (RA). Blood 68(suppl 1):194a, 1986.
50. FITZGERALD DJ, BARRETT JC, and NETTESHEIM P. Changing responsiveness to all-*trans* retinoic acid of rat tracheal epithelial cells at different stages of neoplastic transformation. Carcinogenesis 7:1715–1721, 1986.
51. SHERMAN MI, GUBLER ML, BARKAI U, ET AL. Role of retinoids in differentiation and growth of embryonal carcinoma cells. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 42–60.
52. LOTAN R. Mechanism of inhibition of tumor cell proliferation by retinoids. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 97–105.
53. MARCELO CL, and MADISON KC. Regulation of the expression of epidermal keratinocyte proliferation and differentiation of the vitamin A analogs. Arch Dermatol Res 276:381–389, 1984.
54. SCHINDLER J, MATTHAEI KI, and SHERMAN MI. Isolation and characterization of mouse mutant embryonal carcinoma cells which fail to differentiate in response to retinoic acid. Proc Natl Acad Sci USA 78:1077–1080, 1981.
55. WANG SY, and GUDAS LJ. Selection and characterizations of F9 teratocarcinoma stem cell mutants with altered responses to retinoic acid. J Biol Chem 259:5899–5906, 1984.
56. SPORN MB, and ROBERTS AB. Role of retinoids in differentiation and carcinogenesis. JNCI 73:1381–1387, 1984.
57. MADEN M, and SUMMERBELL D. Retinoic acid-binding protein in the chick limb bud: identification at developmental stages and binding affinities of various retinoids. J Embryol Exp Morphol 97:239–250, 1986.
58. TICKLE C, ALBERTS B, WOLPERT L, ET AL. Local application of retinoic acid to the limb bond mimics the action of polarizing region. Nature 296:564–566, 1982.
59. SPORN MB, ROBERTS AB, ROCHE NS, ET AL. Mechanism of action of retinoids. Supplement to J Am Acad Dermatol (Topical retinoids: an update. Proceedings of a symposium) 15 (part 2):756–764, 1986.
60. STRICKLAND S, BREITMAN TR, FRICKEL F, ET AL. Structure-activity relationships of a new series of retinoidal benzoic acid derivatives as measured by induction of differentiation of murine F9 teratocarcinoma cells and human HL-60 promyelocytic leukemia cells. Cancer Res 43:5268–5272, 1983.
61. MOON RC, GRUBBS CJ, and SPORN MB. Inhibition of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis by retinyl acetate. Cancer Res 36:2626–2630, 1976.
62. ZILE MH, CULLUM ME, ROLTSCH IA, ET AL. Effect of moderate vitamin A supplementation and lack of dietary vitamin A on the development of mammary tumors in female rats treated with low carcinogenic dose levels of 7,12-dimethylbenz(a)anthracene. Cancer Res 46:3495–3503, 1986.
63. THOMPSON HJ, HERBST EJ, and MEEKER LD. Chemoprevention of mammary carcinogenesis: a comparative review of the efficacy

of a polyamine antimetabolite, retinoids, and selenium. JNCI 77: 595–598, 1986.
64. MCCORMICK DL, SOWELL ZL, THOMPSON CA, ET AL. Inhibition by retinoid and ovariectomy of additional primary malignancies in rats following surgical removal of the first mammary cancer. Cancer 51:594–599, 1983.
65. MCCORMICK DL, MEHTA RG, THOMPSON CA, ET AL. Enhanced inhibition of mammary carcinogenesis by combined treatment with *N*-(4-hydroxyphenyl) retinamide and ovariectomy. Cancer Res 42: 508–512, 1982.
66. MARSH W, and CENTER MS. Dimethylsulfoxide, retinoic acid and 12-*O*-tetra-decanoylphorbol-13-acetate induce a selective decrease in the phosphorylation of P150, a surface membrane phosphoprotein of HL60 cells resistant to Adriamycin. Biochem Biophys Res Commun 138:9–16, 1986.
67. MCCORMICK DL, and MOON RC. Influence of delayed administration of retinyl acetate on mammary carcinogenesis. *In* Proceedings of the 13th International Cancer Congress, p 682, 1982.
68. JETTEN AM. Modulation of cell growth by retinoids and their possible mechanisms of action. Fed Proc 43:134–139, 1984.
69. RISCOE MK, FERRO AJ, and FITCHEN JH. Methylthioadenosine (MTA) metabolism is essential for differentiation of HL-60 cells. Blood 68 (suppl 1):192a, 1986.
70. RUSSELL DH. Ornithine decarboxylase: a key regulatory enzyme in normal and neoplastic growth. Drug Metab Rev 16:1–88, 1985.
71. CONNOR MJ, LOWE NJ, BREEDING JH, ET AL. Inhibition of ultraviolet-B skin carcinogenesis by all-*trans*-retinoic acid regimens that inhibit ornithine decarboxylase induction. Cancer Res 43:171–174, 1983.
72. RUSSELL DH, and HADDOX MK. Antiproliferative effects of retinoids related to the cell cycle-specific inhibition of ornithine decarboxylase. Ann NY Acad Sci 359:281–297, 1981.
73. SCOTT KFF, MEYSKENS FL, JR, and RUSSELL DH. Retinoids increase transglutaminase activity and inhibit ornithine decarboxylase activity in Chinese hamster ovary cells and in melanoma cells stimulated to differentiate. Proc Natl Acad Sci USA 79:4093–4097, 1982.
74. LOPRINZI CL, VERMA AK, BOUTWELL RK, ET AL. Inhibition of phorbol ester-induced human epidermal ornithine decarboxylase activity by oral compounds: a possible role in human chemoprevention studies. J Clin Oncol 3:751–757, 1985.
75. LICHTI U, and YUSPA SH. Inhibition of epidermal terminal differentiation and tumor promotion by retinoids. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 77–89.
76. VERMA AK, and BOUTWELL RK. Vitamin A acid (retinoic acid) a potent inhibitor of 12-*O*-tetradecanoyl-phorbol-13-acetate-induced ornithine decarboxylase activity in mouse epidermis. Cancer Res 37:2196–2201, 1977.
77. VERMA AK, SHAPAS BG, RICE HM, ET AL. Correlation of the inhibition by retinoids of tumor promotor-induced mouse epidermal ornithine decarboxylase activity and of skin tumor promotion. Cancer Res 39:419–425, 1979.
78. BOUTWELL RK, and VERMA AK. The influence of retinoids on polyamine and DNA synthesis in mouse epidermis. Ann NY Acad Sci 359:275–280, 1981.
79. DAVIS PJ, MURTAUGH MP, MOORE WT, JR, ET AL. Retinoic acid-induced expression of tissue transglutaminase in human promyelocytic leukemia (HL-60) cells. J Biol Chem 260:5166–5176, 1985.
80. MURTAUGH MP, and DAVIES PJA. Transglutaminase expression and fibronectin cross-linking on the cell surface of peritoneal macrophages. J Cell Biol 99:326a, 1984.
81. YUSPA SH, and LICHTI U. Retinoids and skin carcinogenesis: a mechanism of anticarcinogenesis by modulation of epidermal differentiation. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 56–65.
82. LUK GK, CIVIN CI, WEISSMAN RM, ET AL. Ornithine decarboxylase: essential in proliferation but not differentiation of human promyelocytic leukemia cells. Science 216:75–77, 1982.
83. JETTEN AM, and SHIRLEY JE. Inhibition of ornithine decarboxylase by retinoic acid and difluoromethylornithine in relation to their effects on differentiation and proliferation. Exp Cell Res 156:221–230, 1985.
84. JETTEN AM, JETTEN MER, SHAPIRO S, ET AL. Characterization of the action of retinoids on mouse fibroblast cell lines. Exp Cell Res 119:289–299, 1979.
85. SUGIURA M, SHAFMAN T, MITCHELL T, ET AL. Involvement of spermidine in proliferation and differentiation of human promyelocytic leukemia cells. Blood 63:1153–1158, 1984.
86. FONTANA JA, REPPUCCI A, DURHAM JP, ET AL. Correlation between the induction of leukemic cell differentiation by various retinoids and modulation of protein kinases. Cancer Res 46:2468–2473, 1986.
87. PLET A, EVAIN D, and ANDERSON WB. Effect of retinoic acid treatment of F9 embryonal carcinoma cells on the activity and distribution of cyclic AMP-dependent protein kinase. J Biol Chem 257:889–893, 1982.
88. LOTAN R, and LOTAN D. Enhancement of melanotic expression in cultured mouse melanoma cells by retinoids. J Cell Physiol 106: 179–189, 1981.
89. LOTAN R, NEUMANN G, and LOTAN D. Characterization of retinoic acid-induced alterations in the proliferation and differentiation of a murine and a human melanoma cell line in culture. Ann NY Acad Sci 359:150–170, 1981.
90. NILES RM, LOEWY B, and ROGEIJ S. The effect of retinoic acid on protein phosphorylation in mouse melanoma cells. Exp Cell Res 164:437–448, 1986.
91. LIVESEY SA, NG KW, COLLIER GR, ET AL. Effect of retinoic acid on cellular content and human parathyroid hormone activation of cyclic adenosine 3′:5′-monophosphate-dependent protein kinase isoenzymes in clonal rat osteogenic sarcoma cells. Cancer Res 45: 5734–5740, 1985.
92. REESE DH, and POLITANO VA. Evidence for the retinoid control of urothelial alkaline phosphatase. Biochem Biophys Res Commun 102:322–327, 1981.
93. BOURGEADE MF, and BESANCON F. Induction of 2′, 5′-oligoadenylate synthetase by retinoic acid in two transformed human cell lines. Cancer Res 44:5355–5360, 1984.
94. MARTNER JE, and BERTRAM JS. Appearance of tyrosine phosphorylation correlates with time of transformation in 10T1/2 cells: inhibition of retinyl acetate. Proc Am Assoc Cancer Res 25:146, 1984.
95. TSAMBAOS D, MERK H, BOLSEN K, ET AL. Arotinoid ethyl ester inhibits induction of aryl hydrocarbon hydroxylase by DMBA. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 129–135.
96. DOGRA SC, KHANDUJA KL, GUPTA MP, ET AL. Effect of vitamin A deficiency on the pulmonary and hepatic drug-metabolizing enzymes in rat. Enzyme 30:99–104, 1983.
97. KUMER S, ANTONY M, and MEHROTRA NK. Oxidized glutathione reductase activity in mouse epidermis: TPA induced change and its modulation by vitamin A. Biochem Biophys Res Commun 125: 967–973, 1984.
98. NAKHASI HL, NAGARAJAN L, and ANDERSON WB. Increase in cell-surface *N*-acetylglucosamide beta (1-4) galactosyltransferase activity with retinoic acid-induced differentiation of F9 embryonal carcinoma cells. FEBS Lett 168:222–226, 1984.
99. SIDELL N, LUCAS CA, and KREUTZBERG GW. Regulation of acetylcholinesterase activity by retinoic acid in a human neuroblastoma cell line. Exp Cell Res 155:305–309, 1984.
100. TSAO MS, NELSON KG, and GRISHAM JW. Biochemical effects of 12-*O*-tetradecanoylphorbol-13-acetate, retinoic acid, phenobarbital, and 5-azacytidine on a normal rat liver epithelial cell line. J Cell Physiol 121:1–6, 1984.
101. HEMMI H, and BREITMAN TR. Induction of NAD^+-glycohydrolase. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 48–54.
102. THOMAS G, CHOMIENNE C, BALITRAND N, ET AL. Granulocytic differentiation induced by retinoic acid in HL-60 cells is associated with changes in adenylate cyclase activity. Anticancer Res 6:857–860, 1986.
103. MERRIMAN RL, and BERTRAM JS. Reversible inhibition by retinoids of 3-methyl-cholanthracene-induced neoplastic transformation in C3H/10T1/2 clone 8 cells. Cancer Res 39:1661–1666, 1979.

104. LOTAN R. Immunomodulatory effects of retinoids. J Nutr Growth Cancer 3:57–65, 1986.
105. MEROMSKY L, and LOTAN R. Modulation by retinoic acid of cellular, surface exposed, and secreted glycoconjugates in cultured human sarcoma cells. JNCI 72:203–215, 1984.
106. NOJIRI H, OHTA M, YOSHIDA M, ET AL. Characteristic shift of glycosphingolipid composition during differentiation of human myeloid leukemic cells and cell lines into granulocytic or macrophage-like mature cells. Exp Hematol 12:418, 1984.
107. REISS M, MANIGLIA CA, GOMEZ JJ, ET AL. Changes in glycosaminoglycan production and cell-cell attachment by all-*trans* retinoic acid in human epidermoid carcinoma cells in vitro. Proc Am Assoc Cancer Res 25:41, 1984.
108. FELSTED RL, GUPTA SK, GLOVER CJ, ET AL. Cell surface membrane protein changes during the differentiation of cultured human promyelocytic leukemia HL-60 cells. Cancer Res 43:2754–2761, 1983.
109. LOTAN R, MEROMSKY L, and MARIKOVSKY Y. Prevention by retinoic acid of anionic site redistribution on the surface of cultured human sarcoma cells. Biol Cell 51:147–156, 1984.
110. BOLMER SD, and WOLF G. Stimulation of fibronectin production by retinoic acid in mouse skin tumors. Cancer Res 42:4465–4472, 1982.
111. BOLMER SD, and WOLF G. Retinoids and phorbol esters alter release of fibronectin from enucleated cells. Proc Natl Acad Sci USA 79:6541–6545, 1982.
112. ZERLAUTH G, and WOLF G. Release of fibronectin is linked to tumor promotion: response of promotable and non-promotable clones of a mouse epidermal cell line. Carcinogenesis 6:73–78, 1985.
113. ZERLAUTH G, and WOLF G. Studies on the tumor promoter-induced release of fibronectin from human lung fibroblasts, and its counteraction by retinoic acid. Carcinogenesis 6:531–534, 1985.
114. LOTAN R, LOTAN D, and MEROMSKY L. Correlation of retinoic acid-enhanced sialyltransferase activity and glycosylation of specific cell surface sialoglycoproteins with growth inhibition in a murine melanoma cell system. Cancer Res 44:5805–5812, 1984.
115. ABITA JP, GAUVILLE C, BALITRAND N, ET AL. Binding of 1251-insulin to the human histiocytic lymphoma cell lines U-937: effect of differentiation with retinoic acid. Leuk Res 8:213–221, 1984.
116. STENDAHL O, ANDERSSON T, COBLE BI, ET AL. Selective modulation of a chemotactic receptor in HL-60 cells by retinoid acid. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 48–54.
117. GRIGORIADIS AE, PETKOVICH PM, ROSENTHAL EE, ET AL. Modulation by retinoic acid of 1,25-dihydroxyvitamin D_3 effects on alkaline phosphatase activity and parathyroid hormone responsiveness in an osteoblast-like osteosarcoma cell line. Endocrinology 119:932–939, 1986.
118. GERCEL C, and SCHROEDER EW. Relationship between cell surface glycoconjugates and cell-cell interactions of murine and human melanoma cells. J Cell Biol 99:63a, 1984.
119. LOTAN R, NEUMANN G, and DEUTSCH V. Identification and characterization of specific changes induced by retinoic acid in cell surface glycoconjugates of S91 murine melanoma cells. Cancer Res 43:303–312, 1983.
120. REISS M, MANIGLIA CA, and SARTORELLI AC. Modulation of cell shedding and glucosaminoglycan synthesis of human malignant keratinocytes by all *trans*-retinoic acid and hydrocortisone in vitro. J Invest Dermatol 86:683–688, 1986.
121. JETTEN AM, and SMITS H. Regulation of differentiation of tracheal epithelial cells by retinoids. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 61–73.
122. REISS M, MANIGLIA CA, and SARTORELLI AC. Reversible effects of retinoic acid on glycosaminoglycan synthesis during differentiation of HL-60 leukemia cells. Cancer Res 45:2092–2097, 1985.
123. DELUCA LM. Studies on mannosyl carrier function of retinol and retinoic acid in epithelial and mesenchymal tissues. J Am Acad Dermatol 6:611–619, 1982.
124. JETTEN AM, and DELUCA LM. Induction of differentiation of embryonal carcinoma cells by retinoid: possible mechanisms. Biochem Biophys Res Commun 114:593–599, 1983.
125. WOLF G, KIORPES TC, MASUCHIGE S, ET AL. Recent evidence for the participation of vitamin A in glycoprotein synthesis. Fed Proc 38:2540–2543, 1979.
126. SHERMAN MI, PATERNOSTER ML, and TAKETO M. Effects of arotinoids upon murine embryonal carcinoma cells. Cancer Res 43:4283–4290, 1983.
127. MOSKAL JR, LOCKNEY MW, MARVEL CC, ET AL. Effect of retinoic acid and phorbol-12-myristate-13-acetate on glycosyltransferase activities in normal and transformed cells. Cancer Res 47: 787–790, 1987.
128. TSAMBAOS D, STADLER R, HILT K, ET AL. Effects of arotinoid ethyl ester on epithelial differentiation and proliferation. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 97–112.
129. MARUYAMA T. Differentiation and the cell membrane excitability of teratocarcinoma OTT 6050 in culture. Dev Biol 108:246–249, 1985.
130. JETTEN AM, BARRET JC, and GILMER TM. Differential response to retinoic acid of Syrian hamster embryo fibroblasts expressing v-src or v-Ha-ras oncogenes. Mol Cell Biol 6:3341–3348, 1986.
131. JETTEN AM, and GOLDFARB RH. Action of epidermal growth factor and retinoids on anchorage-dependent and -independent growth of nontransformed rat kidney cells. Cancer Res 43:2094–2099, 1983.
132. JETTEN AM. Effects of retinoic acid on the binding and mitogenic activity of epidermal growth factor. J Cell Physiol 110:235–240, 1982.
133. DELARCO JE, and TODARO GJ. Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci USA 75:4001–4005, 1978.
134. VANZOOLEN EJJ, VANOOSEWAARD TMJ, and DELAAT SW. Transforming growth factor-B and retinoic acid modulate phenotypic transformation of normal rat kidney cells induced by epidermal growth factor and platelet-derived growth factor. J Biol Chem 261:5003–5009, 1986.
135. ROBERTS AB, ANZANO MA, LAMB LC, ET AL. Antagonistic actions of retinoic acid and dexamethasone or anchorage-independent growth and epidermal growth factor binding of normal rat kidney cells. Cancer Res 44:1635–1641, 1984.
136. LIN JC, and SMITH MC. Antagonistic action of retinoic acid against teleocidin and 12-*O*-tetradecanoylphorbol-13-acetate on activation of Epstein-Barr virus genomes. Carcinogenesis 5:483–487, 1984.
137. ROBERTS AB, FROLIK CA, ANZANO MA, ET AL. Transforming growth factors from neoplastic and nonneoplastic tissues. Fed Proc 42:2621–2626, 1983.
138. CHERINGTON PV, SMITH BL, and PARDEE AB. Loss of epidermal growth factor requirement and malignant transformation. Proc Natl Acad Sci USA 76:3937–3941, 1979.
139. KING LE, JR. What does epidermal growth factor do and how does it do it? J Invest Dermatol 84:165–167, 1985.
140. LOCKYER JM, BOWDEN GT, KELLEY MA, ET AL. Retinoic acid-induced changes in epidermal growth factor binding and in biological responses mediated by phorbol ester tumor promoter. Cancer Lett 22:171–182, 1984.
141. NISHIZUKA Y. Studies and perspectives of protein kinase C. Science 233:305–312, 1986.
142. JETTEN AM, and SHIRLEY JE. Action of retinoic acid on the diacylglycerol-induced ornithine decarboxylase activity, reduction of EGF binding and protein kinase C activation in rat tracheal epithelial 2C5 cells. Exp Cell Res 166:519–525, 1986.
143. TOBLER A, DAWSON MI, and KOEFFLER HP. Retinoids: structure-function relationship in normal and leukemic hematopoiesis in vitro. J Clin Invest 78:303–309, 1986.
144. EARP HS, LEE LW, RAYMOND VW, ET AL. (Abstr) EGF and retinoic acid stimulate EGF receptor synthesis. J Cell Biochem Suppl 10C:129, 1986.
145. MEHTA RG, CERNY WL, and MOON RC. Nuclear interactions of retinoic acid-binding protein in chemically induced mammary adenocarcinoma. Biochem J 208:731–736, 1982.
146. CHYTIL F, and ONG DE. Cellular retinoid-binding proteins. *In* The Retinoids (Sporn MB, Roberts AB, and Goodman DS, eds). San Diego, Academic Press, 1984, vol 2, pp 90–123.
147. WANG SY, and GUDAS LJ. Selection and characterization of F9

teratocarcinoma stem cell mutants with altered responses to retinoic acid. J Biol Chem 259:5899–5906, 1984.
148. PLET A, GERBAUD P, SHERMAN MI, ET AL. Retinoic acid effect on cyclic AMP-dependent protein kinases in embryonal carcinoma cells: studies with differentiation-defective sublines. J Cell Physiol 127:341–347, 1986.
149. LIBBY PR, and BERTRAM JS. Lack of intracellular retinoid-binding proteins in retinol-sensitive cell line. Carcinogenesis 3:481–484, 1982.
150. MATTHAEI KI, ANDREWS PW, and BRONSON DL. Retinoic acid fails to induce differentiation in human teratocarcinoma cell lines that express high levels of a cellular receptor protein. Exp Cell Res 143:471–474, 1983.
151. FUCHS E, and GREEN H. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell 25:617–625, 1981.
152. ECKERT RL, and GREEN H. Cloning of cDNAs specifying vitamin A-responsive human keratins. Proc Natl Acad Sci USA 81:4321–4325, 1984.
153. LEVINE RA, LAROSA GJ, and GUDAS LJ. Isolation of cDNA clones for genes exhibiting reduced expression after differentiation of murine teratocarcinoma stem cells. Mol Cell Biol 4:2142–2150, 1984.
154. CHOU RH, CHERVENICK PA, and BARCH DR. Appearance of a new nucleosomal protein during differentiation of human leukemia (HL-60) cells. Science 223:1420–1423, 1984.
155. WANG SY, LAROSA GJ, and GUDAS LJ. Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyryl AMP in cultured mouse teratocarcinoma cells. Dev Biol 107:75–86, 1985.
156. MURTAUGH MP, DENNISON O, STEIN JP, ET AL. Retinoic acid-induced gene expression in normal and leukemic myeloid cells. J Exp Med 163:1325–1330, 1986.
157. WESTIN EH, WONG-STAAL F, GELMANN EP, ET AL. Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc Natl Acad Sci USA 79:2490–2494, 1982.
158. BENTLEY DL, and GROUDINE M. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL-60 cells. Nature 321:702–706, 1986.
159. CRAIG RW, MAUE RJ, HROMCHAK A, ET AL. Decline of C-MYB expression in human myeloblastic leukemia (ML-1) cells induced to differentiate with daunorubicin, conditioned medium, or retinoic acid. Proc Am Assoc Cancer Res 25:64, 1984.
160. THIELE CJ, REYNOLDS CP, and ISRAEL MA. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 313:404–406, 1985.
161. AMATRUDA TT, III, SIDELL N, RANYARD J, ET AL. Retinoic acid treatment of human neuroblastoma cells is associated with decreased N-myc expression. Biochem Biophys Res Commun 126: 1189–1195, 1985.
162. GIESE NA, NEARY KE, LEVINE N, ET AL. Inhibition by retinoic acid of murine retrovirus-induced cellular transformation and tumor formation. JNCI 74:1135–1144, 1985.
163. PREISLER HD, LI YQ, LAM FM, ET AL. (Abstr) 13 *cis* Retinoic acid and dihydroxyvitamin D_3 alter the regrowth rate of myeloid cells in patients with chronic myelogenous leukemia (CML). Blood 68 (suppl 1):228a, 1986.
164. LAWRENCE HJ, HACK FM, ALHADEFF M, ET AL. Effects of retinoic acid on oncogene expression and clonogenic growth of human myeloid leukemia cells. Clin Res 34:40A, 1986.
165. SEGAL S, and KHOURY G. Differentiation as a requirement for simian virus-40 gene expression in F-9 embryonal carcinoma cells. Proc Natl Acad Sci USA 76:5611–5615, 1979.
166. HOWE CC, LUGG DK, and OVERTON GC. Post-transcriptional regulation of the abundance of mRNAs encoding alpha-tubulin and a 94,000-Dalton protein in teratocarcinoma-derived stem cells versus differentiated cells. Mol Cell Biol 4:2428–2436, 1984.
167. SCHRODER EW, RAPAPORT E, and BLACK PH. Retinoids and cell proliferation. Cancer Surveys 2:223–241, 1983.
168. LOTAN R, LOTAN D, and KADOURI A. Comparison of retinoic acid effects on anchorage-dependent growth, anchorage-independent growth and fibrinolytic activity of neoplastic cells. Exp Cell Res 141:79–86, 1982.
169. BRINCKERHOFF CE, MCMILLAN RM, DAYER JM, ET AL. Inhibition by retinoic acid of collagenase production in rheumatoid synovial cells. N Engl J Med 303:432–436, 1980.
170. BRINCKERHOFF CE, COFFEY JW, and SULLIVAN AC. Inflammation and collagenase production in rats with adjuvant arthritis reduced with 13-*cis*-retinoic acid. Science 221:756–757, 1983.
171. GOLDSTEIN SM, MOSKOWITZ MA, and LEVINE L. Inhibition of stimulated prostaglandin biosynthesis by retinoic acid in smooth muscle cells. Cancer Res 44:120–125, 1984.
172. ECCLES S. Effects of retinoids on growth and dissemination of malignant tumours: immunological considerations. Biochem Pharmacol 34:1599–1610, 1985.
173. DENNERT G. Immunostimulation by retinoic acid. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 117–126.
174. SHAPIRO PE, and EDELSON RL. Effects of retinoids on the immune system. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 225–235.
175. TACHIBANA K, SONE S, TSUBURA E, ET AL. Stimulatory effect of vitamin A on tumoricidal activity of rat alveolar macrophages. Br J Cancer 49:343–348, 1984.
176. HEMILA H, and WIKSTROM M. Retinoids activate superoxide production by polymorphonuclear leucocytes. Scand J Immunol 21: 227–234, 1985.
177. BADWEY JA, ROBINSON JM, CURNETTE JT, ET AL. Retinoids stimulate the release of superoxide by neutrophils and change their morphology. J Cell Physiol 127:223–228, 1986.
178. DENNERT G, CROWLEY C, KOULA J, ET AL. Retinoic acid stimulation of the induction of mouse killer T cells in allogeneic and syngeneic systems. JNCI 62:89–92, 1979.
179. LOTAN R, and DENNERT G. Stimulatory effects of vitamin A analogs on induction of cell-mediated cytotoxicity in vivo. Cancer Res 39:55–58, 1979.
180. EREMIN O, ASHBY J, and RHODES J. Inhibition of antibody-dependent cellular cytotoxicity and natural cytotoxicity by retinoic acid. Int Arch Allergy Appl Immunol 75:2–7, 1984.
181. SIDELL N, RIEBER P, and GOLUB SH. Immunological aspects of retinoids in humans. 1. Analysis of retinoic acid enhancement of thymocyte responses to PHA. Cell Immunol 87:118–125, 1984.
182. TOMITA Y, HIMENO K, NOMOTO K, ET AL. Vitamin A and tumor immunity. Experientia 41:92–93, 1985.
183. ECCLES SA, BARNETT SC, and ALEXANDER P. Inhibition of growth and spontaneous metastasis of syngeneic transplantable tumors by an aromatic retinoic acid analogue. 1. Relationship between tumour immunogenicity and responsiveness. Cancer Immunol Immunother 19:109–114, 1985.
184. ABB J, and DEINHARDT F. Effects of retinoic acid on the human lymphocyte response to mitogens. Exp Cell Biol 48:169–179, 1980.
185. COPE FO, HOWARD BD, and BOUTWELL RK. The in vitro characterization of the inhibition of mouse brain kinase-C by retinoids and their receptors. Experientia 42:1023–1027, 1986.
186. ANDERSON WB, THOMAS TP, PLET A, ET AL. Effect of retinoic acid on cyclic AMP-dependent and calcium, phospholipid-dependent protein kinase activities: counteraction to phorbol ester tumor promoter. *In* Retinoids: New Trends in Research and Therapy (Saurat JH, ed). Basel, Karger, 1985, pp 40–47.
187. O'BRIAN CA, LISKAMP RM, SOLOMON DH, ET AL. Triphenylethylenes: a new class of protein kinase C inhibitors. JNCI 76: 1243–1246, 1986.
188. DAWSON MI, HOBBS PD, CHAN R, ET AL. Conformational restrictions of the retinoid skeleton. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 6–28.
189. BERTRAM JS. Structure-activity relationships among various retinoids and their ability to inhibit neoplastic transformation and to increase cell adhesion in the C3H/10T1/2 CL8 cell line. Cancer Res 40:3141–3146, 1980.
190. SPORN MB, and ROBERTS AB. Biological methods for analysis and assay of retinoids—relationships between structure and activity. *In* The Retinoids (Sporn MB, Roberts AB, and Goodman DS, eds). Orlando, Academic Press, 1984, vol I, pp 235–279.
191. STEPHENS-JARNAGIN A, MILLER DA, and DELUCA HF. The

growth supporting activity of a retinoidal benzoic acid derivative and 4, 4-difluororetinoic acid. Arch Biochem Biophys 235:11-16, 1985.
192. BOLLAG W. Therapeutic effects of an aromatic retinoic acid analog on chemically induced skin papillomas and carcinomas of mice. Eur J Cancer 10:731-737, 1974.
193. BOLLAG W. Prophylaxis of chemically induced benign and malignant epithelial tumors by vitamin A acid (retinoic acid). Eur J Cancer 8:689-693, 1972.
194. CLAMON GH, SPORN MB, SMITH JM, ET AL. Alpha- and beta-retinyl acetate reverse metaplasias of vitamin A deficiency in hamster trachea in organ culture. Nature 250:64-66, 1974.
195. KYRITSIS A, JOSEPH G, and CHADER GJ. Effects of butyrate, retinol, and retinoic acid on human Y-79 retinoblastoma cells growing in monolayer cultures. JNCI 73:649-654, 1984.
196. SIDELL N, ALTMAN A, HAUSSLER MR, ET AL. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. Exp Cell Res 148:21-30, 1983.
197. SIDELL N, and HORN R. Properties of human neuroblastoma cells following induction by retinoic acid. Prog Clin Biol Res 175:39-53, 1985.
198. REISS M, PITMAN SW, and SARTORELLI AC. Modulation of the terminal differentiation of human squamous carcinoma cells in vitro by all-*trans*-retinoic acid. JNCI 74:1015-1023, 1985.
199. OLSSON L, BEHNKE O, and SRENSEN HR. Modulatory effects of 5-azacytidine, phorbol ester, and retinoic acid on the malignant phenotype of human lung cancer cells. Int J Cancer 35:189-198, 1985.
200. KIKUCHI Y, MOMOSE E, KIZAWA I, ET AL. Characterization of an established cell line from human immature teratoma of the ovary and effects of retinoic acid on cell proliferation. Cancer Res 44: 2952-2958, 1984.
201. OHTA M, FURUKAWA Y, IDE C, ET AL. Establishment and characterization of four human monocytoid leukemia cell lines (JOSK-I, -S, -M and -K) with capabilities of monocyte macrophage lineage differentiation and constitutive production of interleukin 1. Cancer Res 46:3067-3074, 1986.
202. WETHERALL NT, and TAYLOR CM. The effects of retinoid treatment and antiestrogens on the growth of T47D human breast cancer cells. Eur J Cancer Clin Oncol 22:53-59, 1986.
203. FRAKER LD, HALTER SA, and FORBES JT. Growth inhibition by retinol of a human breast carcinoma cell line in vitro and in athymic mice. Cancer Res 44:5757-5763, 1984.
204. TAYLOR CM, BLANCHARD B, and WETHERALL NT. The antiproliferative effects of synthetic retinoids and arotinoids on human ovarian and breast cancer cell lines. Proc Am Assoc Cancer Res 25:360, 1984.
205. MOON RC, MEHTA RG, and MCCORMICK DL. Retinoids and mammary gland differentiation. *In* Retinoids, Differentiation and Disease (Nugent J, and Clark S, eds). London, Pitman, 1985, pp 156-167.
206. VERMA AK, RICE HM, SHAPAS BG, ET AL. Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity in mouse epidermis by vitamin A analogs (retinoids). Cancer Res 38:793-801, 1978.
207. ELIAS PM, and WILLIAMS ML. Retinoids, cancer, and the skin. Arch Dermatol 117:160-180, 1981.
208. LASNITZKI I, and GOODMAN DS. Inhibition of the effects of methylcholanthrene on mouse prostate organ culture in vitamin A and its analogs. Cancer Res 34:1564-1571, 1974.
209. CHATTERJEE M, and BANERJEE MR. *N*-Nitrosodiethylamine-induced nodule-like alveolar lesion and its prevention by a retinoid in BALB/c mouse mammary glands in the whole organ in culture. Carcinogenesis 3:801-804, 1982.
210. BRINCKERHOFF CE, NAGASE H, NAGEL JE, ET AL. Effects of all-*trans*-retinoic acid (retinoic acid) and 4-hydroxyphenylretinamide on synovial cells and articular cartilage. J Am Acad Dermatol 6:591-602, 1982.
211. NEWTON DL, HENDERSON WR, and SPORN MB. Structure-activity relationships in hamster tracheal organ culture. Cancer Res 40:3413-3425, 1980.
212. BLISS CI, and ROELS OA. The Vitamins: Chemistry, Physiology, Pathology, Methods, 2nd ed (Gyorgy P, and Pearson WN, eds). New York, Academic Press, vol VI, 1967, pp 197-210.
213. SIESTSEMA WK, and DELUCA HF. A new vaginal smear assay for vitamin A in rats. J Nutr 112:1481-1489, 1982.
214. FRASCA JM, and GARFINKEL L. 13-*cis*-Retinoic acid and murine pulmonary adenomas: a preliminary report. Nutr Cancer 3:72-74, 1981.
215. GOMEZ EC. Actions of isotretinoin and etretinate on the pilosebaceous unit. J Am Acad Dermatol 6:746-750, 1982.
216. MCGAUGHEY C, JENSEN JL, and TAGHDIRI K. Hamster cheek pouch as a bioassay system in short term screening for tumor initiating and promoting agents. Optimization of parameters and observations of effects of cytotoxicity on tumor promotion by retinyl acetate. J Environ Pathol Toxicol Oncol 5:283-297, 1984.
217. WETHERALL NT, MITCHELL WM, and HALTER SA. Antiproliferative effect of vitamin A on xenotransplanted C_aM_a-15 cells. Cancer Res 44:2393-2397, 1984.
218. LEVINE N. Effects of topical retinoic acid on intracutaneously implanted S91 melanoma in mice. J Invest Dermatol 85:89-92, 1985.
219. WILLHITE CC, and SHEALY YF. Amelioration of embryotoxicity by structural modification of the terminal group of cancer chemopreventive retinoids. JNCI 73:689-695, 1984.
220. CHYTIL F. Retinoic acid: biochemistry and metabolism. J Am Acad Dermatol 15:741-747, 1986.
221. BALOGH-NAIR V, and NAKANISHI K. Retinylnitrones: a new class of retinoids with chemopreventive action. Pharmacol Res 17:93-95, 1984.
222. WILLHITE CC, DAWSON MI, and WILLIAMS KJ. Structure-activity relationships of retinoids in developmental toxicology. I. Studies on the nature of the polar terminus of the vitamin molecule. Toxicol Appl Pharmacol 74:397-410, 1984.
223. YEN A, POWERS V, and FISHBAUGH J. Retinoic acid induced HL-60 myeloid differentiation: dependence of early and late isomeric structure. Leuk Res 10:619-629, 1986.
224. FABIAN I, and SHVARTZMAYER S. Effect of a new retinoidal benzoic acid derivative on normal human hematopoietic progenitor cell growth in vitro. Cancer Res 46:2413-2415, 1986.
225. LOTAN R, STOLARSKY T, and LOTAN D. Relationships between the structure of retinoidal benzoic acid derivatives and their ability to inhibit the proliferation of melanoma cells in culture. J Nutr Growth Cancer 1:71-76, 1983.

BRIEF REPORTS

Inefficacy of Cisplatin and Etoposide as Salvage Therapy for Children With Recurrent or Unresponsive Soft Tissue Sarcoma[1,2,3]

R. Beverly Raney, Jr[4,*]

Cisplatin (CPDD) and etoposide (VP-16) have activity as single agents in children with recurrent soft tissue sarcoma, with response rates approximating 20%. Sixteen evaluable patients with soft tissue sarcoma, 0.9 to 16 years of age, were treated with a combination of CPDD (75–100 mg/m^2 iv × 1) and VP-16 (100–150 mg/m^2/day iv × 3–5) every 3 weeks after recurrence or failure to respond to standard therapy. Toxicity consisted of vomiting and myelosuppression. There were no complete responses, and only two children (12.5%) had partial responses lasting 6 weeks. Every patient died of progressive tumor. CPDD and VP-16, as given on this study, had minimal activity. [Cancer Treat Rep 71:407–408, 1987]

The outlook for children with soft tissue sarcoma recurrent after, or unresponsive to, multimodal management with surgery; radiation therapy; vincristine, dactinomycin, and cyclophosphamide (VAC), and doxorubicin (Adr) is very poor. Only five of 90 such children treated after recurrence were alive at the time of one report (1). An effective program for re-treatment of patients with recurrent sarcoma is needed.

Cisplatin (CPDD) and etoposide (VP-16) have some activity against soft tissue sarcoma in children (2,3) and have different mechanisms of action and side effects. This report describes a retrieval program using VP-16 and CPDD in 16 children with recurrent or unresponsive soft tissue sarcoma.

MATERIALS AND METHODS

Patients.—All children with recurrent or unresponsive soft tissue sarcoma were initially treated at the Children's Hospital of Philadelphia from 1975 through 1981. All histologic types of soft tissue sarcoma were considered eligible. The protocol required (*a*) known resistance to VAC and Adr; (*b*) presence of measurable residual tumor; (*c*) written informed consent; and (*d*) no previous treatment with CPDD or VP-16.

Drugs.—CPDD was administered in hospital at 75–100 mg/m^2 iv over 8 hours. Furosemide and mannitol were given as needed to maintain urine output. VP-16 was administered at 125–150 mg/m^2/day iv over 30 minutes for 3–5 successive days, beginning on the day before or the day of CPDD administration. Both drugs were readministered every 3 weeks for at least two cycles, unless limiting toxicity or progressive tumor growth occurred. Radiation therapy and operative management were prohibited during this drug trial until after evaluation was completed.

Pretreatment staging and evaluation of response.—All patients had complete physical examination, bone marrow aspiration, audiogram, and appropriate radiographic and blood studies. Response was evaluated at 3-week intervals by repeating the pertinent studies. Complete response (CR) signified no detectable tumor. Partial response (PR) signified reduction in tumor masses by $\geq$ 50%.

RESULTS

From August 1981 through April 1985, 18 eligible patients were entered on study. Two were later deemed inevaluable because of uncertain tumor type or sudden death due to intracranial metastases. The histologic types of tumor were embryonal rhabdomyosarcoma or neurogenic sarcoma in four patients each; fibrosarcoma, synovial sarcoma, or undifferentiated sarcoma in two

[1] Received Apr 4, 1986; revised Oct 2, 1986; accepted Dec 12, 1986.

[2] Supported in part by Public Health Service grants CA-19372 and CA-14489 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[3] Presented in part at the Fifth Annual Seminar of Cancer Research in Virginia, Norfolk, VA, Mar 23, 1985.

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patients each; and alveolar rhabdomyosarcoma or epithelioid sarcoma in one patient each. The 16 evaluable patients were 0.9–16 years old (median, 11.5) at the time of study entry. All 16 patients had previously received VAC and Adr on a regimen of the Intergroup Rhabdomyosarcoma Study (4) or similar program, and 13 had received radiation therapy to the primary tumor in doses $\geq$ 4000 rad in 4–5 weeeks. Fourteen had relapsed while receiving chemotherapy, and two developed recurrence 1 year after completion of chemotherapy. Ten patients had recurrence after an initial CR; the other six, all irradiated, had never responded completely. Each patient had measurable residual sarcoma at the time of entry in the site of the initial primary tumor (seven patients); the lungs (four); the peritoneum (two); or bones, paraspinal muscles, or scalp (one each).

All 16 patients received the initial cycle of drugs as scheduled. Two had evidence of progressive disease after one cycle and were removed from study. The other 14 received two to five cycles of CPDD and VP-16 (median, two).

Toxicity consisted of vomiting in all patients and myelosuppression in 15. Severe neutropenia ($<$ 500 neutrophils/mm^3) occurred in nine patients (56%), thrombocytopenia ($<$ 50,000 cells/mm^3) in eight (50%), and anemia (hemoglobin, 7–9 g/dl) in nine (56%).

The response rate was disappointing: no one had a CR; only two patients achieved PRs lasting 6 weeks in each patient. Overall, only one patient of the seven with rhabdomyosarcoma or undifferentiated sarcoma developed a PR, and only one of nine with other types of sarcoma had a PR. All 16 patients died of progressive tumor. Median survival after initiation of CPDD and VP-16 was 30 weeks (range, 15–188). The longest survivor had a very slowly growing synovial sarcoma.

DISCUSSION

The lack of efficacy observed with VP-16 and CPDD as given to these children may be due to inadequate dosage of one or both agents or to insensitivity of the tumors. The degree of myelosuppression suggests that adequate doses were given.

Russell and Maurer (personal communication) found that of four children with recurrent rhabdomyosarcoma given CPDD and VP-16, one had a PR and the other two had CRs lasting 5 and 11 months, respectively. Carli et al (5) found that of 21 children with rhabdomyosarcoma, three had CRs lasting 8–11 months and four had PRs. None of five patients with soft tissue sarcomas other than rhabdomyosarcoma responded. Their overall response rate of 27% (seven of 26 patients) appears higher than that of 12.5% (two of 16) found in our patients, but the difference is insignificant (chi-square = 0.52; $P > 0.05$). Drug schedules and toxic effects were similar to those in our study.

Since six of our 16 children had failed to respond completely to initial management, they may have represented a more unfavorable group (38% incomplete responders) than those treated by others. Nevertheless, CPDD and VP-16, as given in this study, were ineffective as salvage therapy, although they may be useful in previously untreated patients (6).

REFERENCES

1. RANEY RB, JR, CRIST WM, MAURER HM, ET AL. Prognosis of children with soft-tissue sarcoma who relapse after achieving a complete response. Cancer 52:44–50, 1983.
2. CHARD RL, JR, KRIVIT W, BLEYER WA, ET AL. Phase II study of VP-16-213 in childhood malignant disease: a Children's Cancer Study Group report. Cancer Treat Rep 63:1755–1759, 1979.
3. BAUM ES, GAYNON P, GREENBERG L, ET AL. Phase II trial of cisplatin in refractory childhood cancer: Children's Cancer Study Group report. Cancer Treat Rep 65:815–822, 1981.
4. MAURER HM, MOON T, DONALDSON M, ET AL. The Intergroup Rhabdomyosarcoma Study: a preliminary report. Cancer 40:2015–2026, 1977.
5. CARLI M, PASTORE G, PERILONGO G, ET AL. (Abstr) Phase II trial of cisplatin (CPDD) and VP-16 in previously treated children with advanced soft tissue sarcomas. *In* Proceedings of the XVIIth Meeting of the International Society of Paediatric Oncology, Venice, Italy, 1985, pp 102–103.
6. RANEY B, CRIST W, RAGAB A, ET AL. Results of intensive treatment of children with advanced soft-tissue sarcoma (STS): an Intergroup Rhabdomyosarcoma Study (IRS)-III pilot program. Proc ASCO 4:246, 1985.

BRIEF REPORTS

Clonidine for Anticipatory Nausea and Vomiting: A Pilot Study Examining Dose-Toxicity Relationships and Potential for Further Study[1,2,3]

John H. Fetting,* Vivian R. Sheidler, Michael E. Stefanek, and John P. Enterline[4,5]

In preparation for studies of noradrenergic activity in anticipatory nausea and vomiting, we performed an open-dose study of clonidine to examine dose-toxicity relationships and indications of antiemetic activity. Nine patients, three each at 0.1, 0.2, and 0.4 mg/day, received clonidine twice a day for 5 days before chemotherapy. Unwanted effects, principally blood pressure reduction, dry mouth, and sedation, accumulated between 4 and 5 μg/kg/day. Four of eight evaluable patients had no anticipatory symptoms on clonidine. It is concluded that clonidine, at a dose of 4 μg/kg/day, might safely probe the role of noradrenergic activity in anticipatory nausea and vomiting. [Cancer Treat Rep 71:409–410, 1987]

Anticipatory nausea and vomiting are classically conditioned but their pathogenesis is unknown (1). Two lines of evidence suggest that noradrenergic activity contributes to anticipatory symptoms. First, an increase in state anxiety has been shown to precede the onset of anticipatory nausea and the antiemetic effect of behavioral interventions is associated with reduced anxiety (2,3). Plasma 3-methoxy-4-hydroxyphenylglycol (MHPG), a metabolite of norepinephrine, is elevated with anxiety, and clonidine, which reduces noradrenergic activity, is anxiolytic (4–6). Second, norepinephrine enhances selective attention to relevant conditioned stimuli (7).

We performed an open-dose pilot study of clonidine to determine: (*a*) if the increased vagal tone associated with nausea and vomiting would exacerbate clonidine's bradycardiac and hypotensive effects producing syncope; (*b*) a well-tolerated dose for future studies; and (*c*) indications of antiemetic activity (8).

METHODS

Nine patients, three each at 0.1, 0.2, and 0.4 mg/day, received clonidine twice a day starting 5 days before chemotherapy. Clonidine was discontinued without tapering (9). All three patients were studied at a dose before recruiting patients for the next higher dose.

Patients with anticipatory symptoms were eligible if they were 18–60 years old, were without cardiovascular disease, were not to receive chlorpromazine, and clearly distinguished their postchemotherapy and anticipatory nausea and vomiting. Patients received their regular postchemotherapy antiemetic(s) but received no other psychotropic for 1 week prior to chemotherapy. All patients were informed about alternative behavioral treatment and gave written consent.

Sitting pulse and blood pressure on clonidine obtained at the time of chemotherapy were compared to pulse and blood pressure obtained during the previous clinic visit. Patients rated a list of side effects they experienced as not present, mild, moderate, or severe by phone on the 2nd and 4th day of clonidine treatment, in the clinic on the day of chemotherapy administration, and by phone on the day after chemotherapy administration (6,10). To facilitate analysis of toxicity, doses were converted to μg/kg/day employing the lesser of actual or ideal body weight.

Just after chemotherapy administration, patients indicated whether they had anticipatory nausea and rated it

[1] Received July 23, 1986; revised Nov 19, 1986; accepted Dec 12, 1986.

[2] Supported in part by American Cancer Society grant PBR-19 and the Anne K. Helmuth Fund. J. H. Fetting is a recipient of an American Cancer Society Clinical Oncology Career Development Award.

[3] This work was a poster presentation at the Meeting of the American Society of Clinical Oncology, Los Angeles, CA, May 1986.

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[5] We thank James Unnerstall, PhD, for his advice in the planning of this study, Stuart A. Grossman, MD, for his comments on an earlier version of this manuscript, and Linda Temple and Linda Richardson for their secretarial assistance.

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on a 100-mm visual analog scale. Anticipatory vomiting was evaluated by the number of vomiting episodes. The durations of nausea and vomiting were the hours between onset of symptoms and chemotherapy administration. On the day after chemotherapy phone inquiry was made about the occurrence of postchemotherapy nausea and vomiting.

RESULTS

Of nine patients studied, seven were women; the median age was 36 years (range, 20–57). Four patients had breast cancer, three had Hodgkin's disease or non-Hodgkin's lymphoma, one had rhabdomyosarcoma, and one had sebaceous gland carcinoma. They had received at least three cycles of chemotherapy and a variety of antiemetics. Eight of nine were outpatients. All patients had anticipatory nausea before the previous cycle of chemotherapy; five had anticipatory vomiting.

Eight of nine patients completed the study. One patient receiving a dose of 0.4 mg/day (5.3 μg/kg/day) withdrew after 48 hours because of multiple toxic effects. The other two patients receiving a dose of 0.4 mg/day required a 50% dose reduction to 0.2 mg/day because of toxicity but completed the study. Patients reported taking clonidine as prescribed.

Syncope did not occur. A $>$ 10 mm Hg reduction in sitting diastolic blood pressure compared to the preclonidine level occurred in one patient receiving 0.2 mg/day (4.6 μg/kg/day) and in the two patients who had their dose reduced from 0.4 mg/day (8.1 and 9.3 μg/kg/day) to 0.2 mg/day (4.1 and 4.7 μg/kg/day). No patient had a pulse $<$ 75 on clonidine.

We focused on moderate and severe side effects which might interfere with compliance in future studies. From one to six moderate or severe side effects were reported by five patients at 0.2 and 0.4 mg/day who were receiving from 4.3 to 9.3 μg/kg/day when these toxic effects were reported. Four patients reported dry mouth; four, drowsiness; two, headache; two, tension; one, dizziness; one, insomnia; one, urinary frequency; and one, trouble in concentrating. No patient reported more than mild dizziness during nausea and/or vomiting.

For this report patients were categorized as having or not having anticipatory nausea and/or vomiting. Two patients at 0.1 mg/day (1.9 and 2.3 μg/kg/day), one patient at 0.2 mg/day (4.3 μg/kg/day), and one patient who had her dose reduced from 0.4 mg/day (9.3 μg/kg/day) to 0.2 mg/day (4.7 μg/kg/day) reported no anticipatory symptoms. All four patients recalled anticipatory symptoms before the previous cycle of chemotherapy. All eight patients who completed clonidine had postchemotherapy nausea and vomiting.

DISCUSSION

This study does not preclude the possibility that increased vagal tone associated with nausea and vomiting exacerbates clonidine's hypotensive and bradycardiac effects. Patients had relatively mild anticipatory symptoms and had falling plasma concentrations during postchemotherapy nausea and vomiting. We would be more reassured if patients had significant nausea and vomiting while at plateau concentrations. However, to the extent that blood pressure reduction and severe toxic effects were produced, this study provides a margin of safety.

The toxicity data suggest a dose for future studies. Reductions in blood pressure and/or moderate and severe side effects, primarily dry mouth and sedation, were seen in patients receiving from 4.1 to 9.3 μg/kg/day. These data suggest that a dose of 4 μg/kg/day, which reduces noradrenergic activity, may be well-tolerated (4).

Whether the lack of anticipatory symptoms reported by four patients resulted from inconsistent occurrence of anticipatory symptoms, a pharmacologic action, placebo effect, or a therapeutic effect of the study procedures is impossible to say.

We are currently employing clonidine to manipulate MHPG in patients with anticipatory symptoms. Finding a significant correlation between MHPG and anticipatory symptoms would suggest that noradrenergic activity is involved in anticipatory symptoms.

REFERENCES

1. REDD WH, and ANDRESEN GV. Conditioned aversion in cancer patients. Behav Therapist 4:3–4, 1981.
2. ANDRYKOWSKI MA, and REDD WH. Longitudinal analysis of the development of anticipatory nausea in cancer chemotherapy. J Consult Clin Psychol 53:447–454, 1985.
3. LYLES JN, BURISH TG, KROZELY MG, ET AL. Efficacy of relaxation training and guided imagery in reducing the aversiveness of cancer chemotherapy. J Consult Clin Psychol 50:509–524, 1982.
4. REDMOND DE, and HUANG Y. New evidence for a locus coeruleus—norepinephrine connection with anxiety. Life Sci 25:2149–2162, 1979.
5. KO GN, ELSWORTH JD, ROTH RH, ET AL. Panic-induced elevation of plasma MHPG levels in phobic-anxious patients: effects of clonidine and imipramine. Arch Gen Psychiatry 40:425–430, 1983.
6. HOEHN-SARIC R, MERCHANT AF, KEYSER ML, ET AL. Effects of clonidine on anxiety disorders. Arch Gen Psychiatry 38:1278–1282, 1981.
7. SARA SJ. Noradrenergic modulation of selective attention: its role in memory retrieval. Ann NY Acad Sci 444:178–193, 1985.
8. PETTINGER WA. Pharmacology of clonidine. J Cardiovasc Pharmacol 2(suppl 1):S21–S28, 1980.
9. WHITSETT TL, CHRYSANT SG, DILLARD CL, ET AL. Abrupt cessation of clonidine administration: a prospective study. Am J Cardiol 41:1285–1290, 1978.
10. KERANEN A, NYKANEN S, and TASKINEN J. Pharmacokinetics and side effects of clonidine. Eur J Clin Pharmacol 13:97–101, 1978.

Clinical Trial of Plasma Perfusion Over Immobilized Staphylococcal Protein A in Metastatic Breast Cancer[1]

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Nineteen patients with metastatic breast cancer refractory to conventional therapy were treated with plasma perfusion over 200 mg of staphylococcal Protein A immobilized on a silica matrix. Fever and chills (33%), pain at the site of tumor (18%), and dyspnea (16%) were the most frequent toxic effects encountered. Four patients (21%) developed a disseminated rash which necessitated cessation of treatment. Of 16 patients evaluable for response, one achieved a minor response of chest wall disease and two had no change in hepatic metastases for 4 and 5 months' duration. Potential mechanisms of antitumor effect are discussed. [Cancer Treat Rep 71:411-413, 1987]

Variable responses have been reported with reinfusion of autologous plasma that has been perfused over immobilized staphylococcal Protein A (SPA) (1-5). We treated 19 patients with metastatic breast cancer refractory to conventional therapy utilizing a standardized column containing immobilized SPA to assess the spectrum of toxicity and evaluate therapeutic efficacy.

MATERIALS AND METHODS

The column utilized a polycarbonate chamber containing an inert silica matrix to which 200 mg of extracted SPA was covalently bound (IMRE Corporation, Seattle, WA). For each treatment, 500 ml of blood was phlebotomized using phosphate-citrate-dextrose solution, cellular elements were reinfused, and plasma was perfused through the column and slowly reinfused. Patients were staged and evaluated by physical examination, photography, and radiography. Twelve treatments were administered unless unacceptable toxicity or disease progression occurred. Minor response was defined as a $< 50\%$ but $> 25\%$ reduction in the product of the largest perpendicular diameter of measurable lesions for a minimum of 30 days without new lesions. No change was defined as no evidence of increase in tumor size for at least 8 weeks and no new lesions. Prior to each treatment, patients had cbc and serum chemistries (SMA-12). Serum IgG levels, C_3 levels (radial immunodiffusion), and Clq binding circulating immune complexes (CIC) (enzyme-linked immunoadsorbent assay) were measured immediately before and after every third treatment.

RESULTS AND DISCUSSION

Toxic effects are shown in table 1. Symptoms generally began within the first half-hour after reinfusion and were resolved within 3 hours. The higher incidence of toxicity compared to a previous trial (5) may be related to the matrix used [silica versus collodion charcoal (5)]. A disseminated maculopapular rash necessi-

TABLE 1.—Clinical toxicity of SPA therapy*

Toxic effect	% of total treatments	No. of patients (%)
Fever and chills	33	17 (89)
Pain at tumor site	18	9 (47)
Dyspnea	16	9 (47)
Nausea and vomiting	10	9 (47)
Arthralgias	7	4 (21)
Myalgias	5	3 (16)
Skin rash	4	5 (26)
Hypotension	2	3 (16)

*165 treatments administered to 19 patients with metastatic breast cancer.

[1] Received July 28, 1986; revised Dec 10, 1986; accepted Jan 12, 1987.

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tated cessation of treatment in four patients (21%). Subsequent reinfusion of 10 ml of perfused plasma in one patient (No. 9) was associated with anaphylaxis (back pain, hypotension, and exacerbation of rash). Thrombocytopenia (70,000 cells/μl) and mild elevation of fibrin degradation products with the rash occurred in one patient (No. 11). Skin biopsies were not obtained. Hepatic and renal functions did not change with therapy. Nonhemolytic normochromic normocytic anemia occurred in four patients (21%), also not previously reported.

Of 16 evaluable patients (table 2), one patient (No. 1) achieved a minor response of chest wall disease for 2 months, and two patients (Nos. 9 and 10) had no change in hepatic metastases for 4 and 5 months' duration, respectively. All patients had low CIC levels (< 10 μg/ml of aggregated human IgG) during the trial. Serum IgG and C_3 levels stayed within the normal range throughout treatment and did not significantly change before and after each treatment. Thus, removal of CICs or IgG with postulated "blocking activity" (6) was unlikely. Of interest, activation of the alternate pathway of complement by SPA-treated sera has been demonstrated in vitro, with inhibition by citrate-based anticoagulation (7). Other immunologic mechanisms are also possible (8,9), since small quantities of SPA may leach off column matrices and into the circulation (10). As used herein, we observed no major objective responses with plasma perfusion over immobilized SPA for patients with metastatic breast cancer; additional studies utilizing this column and dose schedule do not appear warranted.

TABLE 2.—Summary of patients and responses to SPA immunoadsorption

Patient No.	Age (yrs)	Estrogen receptor status	Prior treatment*	Response to prior treatment†	Location of metastases	No. of treatments	Frequency of treatments	Response (mos)†
1	49	Negative	FAC, VLB, XRT, rIFN, Mito-C	PR (VLB)	Chest wall	12	1/wk	MR (2)
2	49	Negative	FAC, VLB, MTX	PD	Bone (lytic)	3	1/wk	PD
3	33	Negative	FAC, CMF, VLB, XRT	MR (FAC) CR (VLB)	Lung	8	1/wk	PD
4	49	Negative	XRT, CAP, VAD, VMF	PR (CAP)	Lung	12	3/wk	PD
5	24	Negative	VACP, XRT, VLB	PD	Bone (lytic)	12	3/wk	PD
6	59	Positive	CAVP-16, XRT, VLB, TMX, MTX	CR (CAVP-16)	Chest wall, axilla	12	3/wk	PD
7	55	Positive	TMX, XRT, CMF, MG, AT, HT, rIFN, AV	PR (MG)	Chest wall, liver	12	3/wk	PD
8	66	Not available	FAC/CMF, XRT, TMX, MG, AT, HT, VLB	CR (AT)	Lung, liver	12	3/wk	PD
9	43	Positive	FAC/TMX, VP-16, MTX, VLB, FUDR, IA CDDP/VLB	CR (FAC/TMX)	Liver	5	3/wk	SD (4)
10	55	Positive	TMX, FAC, IA VLB	PD	Liver	12	3/wk	SD (5)
11	61	Positive	FAC, MTX/VLB/VP-16	PR (FAC)	Chest wall	1	3/wk	Inevaluable
12	50	Not available	CMF, TMX, AV	PD	Chest wall, liver	12	3/wk	PD
13	53	Positive	CMF/TMX, AT, AV, Mito-C, 5-FU	Stable (AV)	Chest wall	12	3/wk	PD
14	69	Not available	FAC, CMF, XRT, AV/TMX, MG	Stable (AV/TMX)	Bone (lytic)	4	3/wk	Inevaluable
15	53	Negative	FACVP, XRT, MTX, Mito-C	PR (FACVP)	Chest wall	7	3/wk	PD
16	55	Not available	FAC/CMFVP, XRT, MTX/VLB/VP-16, MG, VLB	PD	Chest wall	12	3/wk	PD
17	66	Negative	CMFVP, FAC, TMX, 5-FU, VLB	PD	Chest wall, cervical nodes	2	3/wk	Inevaluable
18	66	Positive	FACVP/TMX, VLB	PR (VLB)	Chest wall, lung	10	3/wk	PD
19	42	Positive	5-FU/Melph, CAVP-16, Mito-C, VLB, MTX/5-FU, TMX, VP-16	PR (VLB) PR (TMX)	Chest wall, cervical nodes	6	3/wk	PD

*FAC = 5-fluorouracil, doxorubicin, and cyclophosphamide; VLB = vinblastine; XRT = radiotherapy; rIFN = recombinant interferon alpha; Mito-C = mitomycin; MTX = methotrexate; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; CAP = cyclophosphamide, doxorubicin, and prednisone or cisplatin; VAD = vincristine, doxorubicin, and dexamethasone; VMF = vincristine, methotrexate, and 5-fluorouracil; VACP = vincristine, doxorubicin, cyclophosphamide, and prednisone; CAVP-16 = cyclophosphamide, doxorubicin, and etoposide; TMX = tamoxifen; MG = megesterol; AT = aminoglutethimide; HT = halotestin; AV = doxorubicin and vinblastine; VP-16 = etoposide; FUDR = floxuridine; IA = intra-arterial (hepatic); CDDP = cisplatin; 5-FU = 5-fluorouracil; FACVP = 5-fluorouracil, doxorubicin, cyclophosphamide, and etoposide; CMFVP = cyclophosphamide, methotrexate, 5-fluorouracil, and etoposide; and Melph = melphalan.

†PR = partial remission; PD = progressive disease; MR = minor response; and CR = complete remission.

REFERENCES

1. TERMAN DS, YOUNG JB, SHEARER WT, ET AL. Preliminary observations of the effects on breast adenocarcinoma of plasma perfused over immobilized protein A. N Engl J Med 305:1195–1200, 1981.
2. BANSEL SC, BANSAL BR, THOMAS HL, ET AL. Ex-vivo removal of serum IgG in a patient with colon carcinoma. Cancer 42:1–18, 1978.
3. HAKANSSON L, JONSSON S, SODERBERG M, ET AL. Tumor regression after extracorporeal affinity chromatography of blood plasma across agarose beads containing staphylococcal protein A. Eur J Clin Oncol 20:1377–1387, 1984.
4. BERTRAM JH, GRUNBERG SM, SHULMAN I, ET AL. Staphylococcal Protein A column: correlation of mitogenicity of perfused plasma with clinical response. Cancer Res 45:4486–4494, 1985.
5. FER MF, BEMAN J, STEVENSON HC, ET AL. A trial of autologous plasma perfusion over protein A in patients with breast cancer. J Biol Response Mod 3:352–358, 1984.
6. HELLSTROM I, HELLSTROM KE, EVANS CA, ET AL. Serum mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor-specific antigens. Proc Natl Acad Sci USA 62:362–368, 1969.
7. NAKANISHI K, ZBAR B, and BOROS T. Plasma therapy of primary rat mammary carcinoma: dependence of consumption of C_3 during absorption of plasma with Sepharose derivatives on the anticoagulant. Cancer Res 45:4122–4127, 1986.
8. SMITH EM, JOHNSON HM, and BLALOCK JE. *Staphylococcus aureus* protein A induces the protection of interferon-alpha in human lymphocytes and interferon-alpha/beta in mouse spleen cells. J Immunol 130:773–776, 1983.
9. FLEISHER B. Induction of T cell growth factor synthesis in human peripheral blood lymphocytes by staphylococcal protein A. J Immunol Methods 47:191–200, 1981.
10. BALINT J, IKEDA Y, LANGONE JJ, ET AL. Tumoricidal response following perfusion over immobilized protein A: identification of immunoglobulin oligomers in serum after perfusion and their partial characterization. Cancer Res 44:734–743, 1984.

BRIEF REPORTS

Respiratory Dyskinesia Due to Antiemetic Therapy in a Cancer Patient[1]

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Cancer patients commonly receive neuroleptics as antiemetics to relieve nausea and vomiting induced by chemotherapeutic agents. Respiratory dyskinesia, an infrequent, acute, neuroleptic-induced, dystonic reaction, is a potentially life-threatening entity that responds promptly to anticholinergic medication. [Cancer Treat Rep 71:415-416, 1987]

Droperidol (DRO) is a butyrophenone (a nonphenothiazine compound) used primarily as a preanesthetic sedative and in neuroleptanalgesia (1). Butyrophenones have been shown to have significant antiemetic activity due to dopamine receptor antagonism and potent inhibition of the chemoreceptor trigger zone; they are thus useful in controlling emesis induced by chemotherapy (2,3). However, as with other butyrophenones and the piperazine phenothiazines, the incidence of extrapyramidal reactions with DRO is greater than with other neuroleptics (4,5). We report a case of DRO-induced respiratory dyskinesia in a cancer patient.

CASE REPORT

A 24-year-old male nonsmoker with acute myelogenous leukemia had previously undergone multiple courses of chemotherapy including intrathecal cytarabine (ARA-C) and iv doxorubicin (ADR), vincristine (VCR), mitoxantrone, 6-mercaptopurine, cyclophosphamide (CTX), carmustine (BCNU), etoposide (VP-16-213), and prednisone (PRED). He was in his fourth remission on admission to The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute in Houston for further chemotherapy and bone marrow transplantation. Vital signs, physical examination, serum chemistries, hemogram, and coagulation studies were normal. Past medical history was negative for any cardiac, pulmonary, neurologic, or endocrine symptomatology.

On the third hospital day the patient received 645 mg of iv BCNU with iv antiemetic medication consisting of 4 mg of DRO, 25 mg of diphenhydramine (DIP), 0.5 mg of lorazepam (LOR), and 10 mg of dexamethasone. The following day he received two iv injections each of CTX (3.225 g) and VP-16-213 (270 mg), preceded by the same antiemetics. In addition, the patient received two doses of iv DRO (2 mg), DIP (25 mg), and LOR (0.5 mg) for nausea and vomiting. He complained of a gasping sensation and moderate anxiety and was given a third dose of the antiemetic combination, with some relief.

During the fifth hospital day the patient received the same antiemetics, CTX, and VP-16-213. Within a brief period of time he complained of the same symptoms and had difficulty breathing. Blood pressure was 150/90; pulse, 120/minute; respirations, 28/minute. There were contractions of the sternocleidomastoid muscles, grunting respirations with intermittent voluntary control of the respiratory distress, facial flushing, marked restlessness, anxiety, and persistent pacing. Initially, the diagnosis of an acute anxiety attack was made and the patient was given iv DIP (50 mg), iv methylprednisolone (100 mg), and an order for 1 mg of LOR orally twice daily. However, careful review of the patient's medical record revealed documentation of a similar reaction 2 years earlier, after he had received DRO alone prior to chemotherapy (ADR, VCR, ARA-C, and PRED). Although an anxiety attack was also considered in the differential then, the diagnosis was thought to be a DRO reaction, and symptoms resolved promptly after the patient received iv DIP. With this information, the diagnosis was amended to acute extrapyramidal reactions of respiratory dyskinesia and akathisia. The pa-

[1] Received Aug 12, 1986; revised Dec 9, 1986; accepted Jan 12, 1987.

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tient was then treated with 2 mg of iv benztropine mesylate (BZM) and had complete symptom resolution. He continued to receive oral LOR and was maintained on 1 mg of BZM three times daily while receiving antiemetics. He experienced no further extrapyramidal reactions.

DISCUSSION

Extrapyramidal reactions are among the most common side effects of the neuroleptics (5). Those reported with DRO have included drowsiness, leg restlessness, and dystonia (1,3,6). Acute dystonias, primarily involving the muscles of the head and neck, occur in approximately 2% of patients receiving neuroleptics. These reactions tend to occur within the first 72 hours of neuroleptic therapy and are episodic, recurrent, dose-dependent, and more common with the higher-potency drugs. They also occur more often in the young and in males (7).

Respiratory dyskinesia is an acute dystonic reaction that may not be recognized as an extrapyramidal reaction but rather as a respiratory side effect, thus leading to underreporting and misdiagnoses of hysteria, anxiety, and psychogenic hyperventilation (8,9). The symptoms are in sharp contrast to the breathing of the hyperventilator, which tends to be barely noticeable and is punctuated by inobtrusive sighs and yawns (8).

Respiratory dysfunction from chemotherapeutic agents such as those administered to this patient has also been reported (10). It is unlikely, however, that the administration of ARA-C, ADR, or VCR caused this patient's respiratory complications, as he had tolerated at least five courses of combined treatment with all three drugs without any respiratory problems. Only when DRO was added to control emesis did these symptoms emerge.

CTX, VP-16-213, and BCNU can cause pulmonary complications; however, these are not common occurrences, especially in a young patient with no predisposition to pulmonary compromise. In addition, the symptoms of respiratory dysfunction that have been reported with the use of these chemotherapeutic agents are quite different from those experienced by the patient.

Akathisia, the subjective feeling of tension and inability to tolerate inactivity (which then gives rise to restless movement), occurs in 20% of patients receiving neuroleptics and is a common extrapyramidal reaction (5). Akathisia occurs early in therapy, is dose-dependent, and is more frequent in women. Frequently misdiagnosed as agitation or anxiety, akathisia is often inadvertently exacerbated by erroneous treatment with more neuroleptics or inadequately treated with benzodiazepines. Akathisia and the acute dystonic reactions respond promptly to anticholinergic medication (7).

The presence of one or more extrapyramidal reactions does not always mean that the offending agent can no longer be used. Frequently, a reduction in dosage or the addition of anticholinergic medication will relieve the adverse symptomatology. Thus, it becomes increasingly important for the prescribing physician to be able to recognize and treat these reactions due to neuroleptics, especially those that can be life-threatening.

REFERENCES

1. LEWIS GO, BERNATH AM, ELLISON NM, ET AL. Double-blind crossover trial of droperidol, metoclopramide, and prochlorperazine as antiemetics in cisplatin therapy. Clin Pharm 3:618-621, 1984.
2. DONOVITZ GS, O'QUINN AG, and SMITH ML. Antiemetic efficacy of high-dose corticosteroids and droperidol in cisplatin-induced emesis: a controlled trial with droperidol and metoclopramide. Gynecol Oncol 18:320-325, 1984.
3. STUART-HARRIS R, STARKE I, BUCKMAN R, ET AL. Chlorpromazine, placebo and droperidol in the treatment of nausea and vomiting associated with cisplatin therapy. Postgrad Med J 59:500-503, 1983.
4. FRYTAK S, and MOERTEL CG. Management of nausea and vomiting in the cancer patient. JAMA 245:393-396, 1981.
5. GELENBERG AJ. Psychoses. *In* The Practitioner's Guide to Psychoactive Drugs, 2nd ed (Bassuk EL, Schoonover SC, and Gelenberg AJ, eds). New York, Plenum Publishing Corporation, 1983, pp 115-165.
6. MARSHALL BE, and WOLLMAN H. General anesthetics. *In* The Pharmacological Basis of Therapeutics, 7th ed (Gilman AG, Goodman LS, and Rall TW, eds). New York, Macmillan Publishing Co, 1985, pp 276-301.
7. CUMMINGS JL. Neuropsychiatric aspects of movement disorders. *In* Clinical Neuropsychiatry. Orlando, Grune & Stratton, Inc, 1985, pp 144-146.
8. CHIANG E, PITTS W, and RODRIGUEZ-GARCIA M. Respiratory dyskinesia: review and case reports. J Clin Psychiatry 46:232-234, 1985.
9. AYD FJ, JR. Respiratory dyskinesias in patients with neuroleptic-induced extrapyramidal reactions. Int Drug Ther Newsletter 14: 1-3, 1978.
10. DORR RT, and FRITZ WL. Cancer Chemotherapy Handbook. New York, Elsevier, 1983, pp 299, 348, 424, 689.

High-Dose Cytarabine in Small Cell Lung Cancer[1]

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The activity of cytarabine (Ara-C) in small cell lung cancer (SCLC) is poorly defined. Two studies employing conventional-dose Ara-C (100–150 mg/m^2/day for 3 days) in 25 patients (four previously untreated) revealed no activity (1,2). In a study of high-dose Ara-C (HDAraC) in refractory SCLC, nine patients received 3 g/m^2, either on two occasions 12 hours apart or as a single 24-hour infusion. There were no responses (3).

We have investigated a more prolonged regimen of HDAraC in 15 patients with evaluable relapsed or refractory SCLC. Twelve patients had previously received only a single regimen of chemotherapy. All patients had normal pretreatment blood cell counts and none had marked bone marrow infiltration. Patient characteristics are shown in table 1.

Ara-C (2 g/m^2) was given twice daily as a 3-hour iv infusion for 2 days, repeated every 3 weeks.

Patients were evaluated following each course of treatment with clinical examination and chest x-ray. Treatment was discontinued in patients with progressive disease or stable disease after two courses of treatment. Response was assessed according to World Health Organization criteria.

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Males	8
Females	7
Median age in yrs (range): 59 (45–68)	
Median Karnofsky performance status: 70 (50–90)	
Prior treatment	
1 combination regimen	8
1 combination regimen and radiotherapy	1
High-dose cyclophosphamide	3
2 combination regimens	2
2 combination regimens and radiotherapy	1
Disease extent*	
Limited	6
Extensive	9
No. of courses of HDAraC	
1	9
2	5
6	1
Response	
Complete	1
Partial	0
Stable disease	3
Disease progression	11
Hematologic toxicity	
Days with neutropenia ($< 1 \times 10^9$ cells/L)	
Mean: 4.4	
Range: 0–11	
Days with thrombocytopenia ($< 50 \times 10^9$ cells/L)	
Mean: 2.4	
Range: 0–5	

*At start of HDAraC.

RESULTS AND TOXICITY

One patient with limited disease (the most heavily pretreated patient) achieved complete remission on chest x-ray lasting 10 weeks before relapse at the primary site and intracerebrally. No partial remissions were achieved. Median survival from the start of treatment was 3.5 months.

Five patients receiving more than one course of treatment required dosage reductions (4-week schedule, two patients; 75% dose, three patients). Admission was required for management of thrombocytopenia or neutropenia following 18 courses of treatment. Platelet transfusions were required following ten courses of treatment. Nausea and vomiting was generally mild and transient.

DISCUSSION

Established drugs achieve high response rates in SCLC, but long-term survival is rare (4). There is a clear need for new agents whose activity is best sought in patients who have not received extensive prior treatment. The single brief response to HDAraC observed in this group of moderately pretreated patients indicates a low level of activity for this drug in SCLC. In contrast, second-line treatment with conventional combination chemotherapy would be expected to achieve response

[1] Received Aug 4, 1986; revised Oct 27, 1986; accepted Dec 5, 1986.

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rates of around 30% in such patients with much less myelotoxicity (5). The marked hematologic toxicity associated with HDAraC and the frequent need for hospitalization of patients whose life span is already short indicate that HDAraC is unlikely to offer significant benefit in SCLC.

REFERENCES

1. O'DONNELL JF, MAURER LH, FORCIER RJ, ET AL. Intensive cytosine arabinoside therapy in small cell carcinoma of the lung. Am J Clin Oncol 7:415–418, 1984.
2. CHIUTEN DF, CARR DT, DHINGRA HM, ET AL. High-dose ara-c given as a continuous infusion or intermittent schedule in lung cancer. Proc ASCO 3:227, 1984.
3. STUART-HARRIS R, BISHOP J, RAGHAVAN D, ET AL. Cytarabine: an inactive drug for extensive-stage small cell lung cancer. Cancer Treat Rep 70:303–304, 1986.
4. DAVIS S, WRIGHT PW, SCHULMAN SF, ET AL. Long-term survival in small cell carcinoma of the lung: a population experience. J Clin Oncol 3:80–91, 1985.
5. DAUGAARD G, HANSEN HH, and RØRTH M. Phase II study of vindesine, cisplatin and hexamethylmelamine (VCH) in small cell carcinoma of the lung. Cancer Treat Rep 68:1179–1181, 1984.

FAM (5-Fluorouracil, Doxorubicin, and Mitomycin) Plus Triazinate (FAM-T) in Gastric Carcinoma: A Combined Phase II Trial of the Mid-Atlantic Oncology Program and the Pan American Health Organization[1,2]

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Gastric adenocarcinoma has moderate sensitivity to combination chemotherapy. The combination of 5-fluorouracil (5-FU), doxorubicin, and mitomycin (FAM) (1,2) is one of the more widely utilized regimens. By integrating the antifol triazinate into the FAM regimen we sought to take advantage of its apparent independent activity in gastric carcinoma (3) and of the antifol/5-FU interaction (4). A phase II trial of the new combination, FAM-T, was carried out simultaneously within the Mid-Atlantic Oncology Program (MAOP) and the National Cancer Institute-Pan American Health Organization Collaborative Cancer Treatment Research Program (PAHO). Thirty-seven patients with measurable metastatic gastric adenocarcinoma were treated with FAM-T. Of 31 fully evaluable patients, only four (13%) achieved responses [one complete (CR) and three partial (PR)]. This contrasts with response rates of the order of 40% reported in multiple studies using FAM alone in similar populations (1,2).

PATIENTS AND METHODS

The FAM-T regimen is shown in table 1. It differs from the original FAM regimen as described by Macdonald et al (1) only in the addition of triazinate (Baker's antifol) at a dose of 200 mg/m^2 infused iv over 30 minutes, 1 hour before the 5-FU on Days 1 and 29.

Patients had pathologically documented metastatic adenocarcinoma of the stomach, without previous chemotherapy, radiotherapy, or prior malignancy. Measurable disease was required. A performance status of 3 or better (Zubrod) and normal marrow function (wbc count of 4000 and platelet count of 100,000/mm^3) were required. Standard response criteria were utilized.

Thirty-eight patients were registered on protocol. One patient's participation was cancelled before treatment was given; five patients were found to be ineligible (prior chemotherapy, four; no measurable disease, one) and were excluded from analysis. Patient characteristics are summarized in table 2.

RESULTS

Of the 31 evaluable patients for whom follow-up data are available, four (13%) achieved remissions (1 CR, 3 PR), with median duration of 261 days (range, 112–406). Median time from initiation of treatment to pro-

TABLE 1.—FAM-T regimen

Treatment	Day 1	8	15	22	29	36	43	50	57
Triazinate* (200 mg/m^2 iv)	X				X				R E
5-FU (600 mg/m^2 iv)	X	X			X	X			P E
Doxorubicin† (30 mg/m^2 iv)	X				X				A T
Mitomycin (10 mg/m^2 iv)	X								

*Infused over 30 mins, 1 hr prior to FAM (ie, 1 hr should elapse between completion of triazinate and administration of FAM).
†Total dose not to exceed 500 mg/m^2.

[1] Received June 26, 1986; revised Nov 18, 1986; accepted Dec 10, 1986.
[2] Supported by contract N01-CM-27391 (Division of Cancer Treatment) and Public Health Service grant CA-33075 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.
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TABLE 2.—Patient characteristics and results*

Characteristic	MAOP	PAHO	Total
No. of study patients			
Registered	26	12	38
Cancelled	1	0	1
Ineligible	2	3	5
Lost to follow-up	0	1	1
Analyzed	23	8	31
Sex			
Male	68	58	65
Female	32	42	35
Age in yrs			
Mean	59		
SD	13		
Range	33–79		
Performance status			
Fully active (0)	21	—	13
Ambulatory (1)	37	16	29
In bed < 50% (2)	37	42	39
In bed > 50% (3)	5	42	19
Response			
PR	13	0	10
CR	4	0	3
CR + PR	17	0	13
Highest toxicity			
None (0)			5
Mild (1)			11
Moderate (2)			22
Severe (3)			53
Life-threatening (4)			8
Lethal (5)			5

*Unless specified otherwise, values = % of patients.

gression was 103 days (range, 28–527) and median survival was 169 days. Mean number of days on treatment was 115 (range, 1–439).

All four of the responders (four of 23; 17%) were in the MAOP group; none of eight PAHO patients responded. This may be in part due to the generally higher performance status of the MAOP population (58% with performance status of 0–1 vs 16% in the PAHO group), but the difference is not significant ($P = 0.2$). Median survival of the MAOP population was 175 days, versus 158 for the PAHO population.

Two treatment-related deaths occurred on this protocol. Grade 3 (severe) and grade 4 (life-threatening) toxicity occurred in 20 (53%) and three (8%) patients, respectively (table 1). Over two-thirds of patients experienced at least mild leukopenia or thrombocytopenia. Both deaths were associated with hematologic toxicity.

DISCUSSION

Two developments prompted the investigation of the combination FAM-T. First, Cadman and co-workers (4) showed that pretreatment with antifolates can potentiate the activity of 5-FU. Second, the antifolate triazinate (at higher dose than used in the FAM-T combination) had activity of its own in gastric cancer, with a response rate of 17% demonstrated in a series of 23 patients (3).

In addition, Klein and co-workers (5) have published results with the combination of 5-FU, doxorubicin, and methotrexate (FAMTX) in gastric carcinoma. Their updated series now reports objective response in 59% of 100 patients, with 12% CR and median survival of 9 months. The FAMTX combination uses a much larger dose of a different antifol. However, the early results with FAMTX lent further rationale to the investigation of the FAM-T combination.

In this series with FAM-T a much lower activity has been noted. With four responses among 31 evaluable patients, the 95% confidence interval for true response rate would be 4%–30%. Thus, from this limited series it would appear unlikely that the true response rate of FAM-T, as administered here, will be significantly better than FAM alone.

REFERENCES

1. MACDONALD JS, SCHEIN PS, WOOLLEY PV, ET AL. 5-Fluorouracil, doxorubicin and mitomycin (FAM) combination chemotherapy for advanced gastric cancer. Ann Intern Med 93:533–536, 1980.
2. O'CONNELL MJ. Current status of chemotherapy for advanced pancreatic and gastric cancer. J Clin Oncol 3:1032–1039, 1985.
3. BRUCKNER HW, LOKICH JJ, and STABLEIN DM. Studies of Baker's antifol, methotrexate, and razoxane in advanced gastric cancer: a Gastrointestinal Tumor Study Group report. Cancer Treat Rep 66: 1713–1717, 1982.
4. CADMAN E, HEIMER R, and DAVIS L. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation for drug synergism. Science 205:1135–1137, 1979.
5. KLEIN HO, WICKRAMANAYAKE PD, and FARROKH GR. 5-Fluorouracil (5-FU), Adriamycin (ADM) and methotrexate (MTX)—a combination protocol (FAMTX) for treatment of metastasized stomach cancer. Proc ASCO 5:84, 1986.

Bleomycin, Cyclophosphamide, and Dactinomycin in Metastatic Osteosarcoma: Lack of Tumor Regression in Previously Treated Patients[1,2]

Charles B. Pratt,* Sidnei Epelman, and Norman Jaffe[3]

Bleomycin, cyclophosphamide, and dactinomycin (BCD) used in combination were reported to cause objective tumor regression in five of five untreated patients and three of eight previously treated patients with metastatic osteosarcoma (1). The responses described in the initial communication were impressive and included reduction in pain and soft tissue swelling due to tumor mass, decrease in level of serum alkaline phosphatase, and reduction in size of pulmonary metastases. Rosen et al (2) later used BCD in the preoperative treatment of patients with osteosarcoma. We report the results of eight patients previously treated with chemotherapy for metastatic or unresectable osteosarcoma who received treatment with BCD at our institutions.

PATIENTS AND METHODS

Patients with measurable indicators of disease activity were treated at St. Jude Children's Research Hospital (six patients) and at M. D. Anderson Hospital (two). All of the patients had received significant amounts of prior chemotherapy (table 1), and the tumors of these patients were considered to be resistant to conventional agents.

Treatment consisted of bleomycin (15 mg/m^2), cyclophosphamide (600 mg/m^2), and dactinomycin (0.6 mg/m^2) administered iv on 2 consecutive days. Treatment was repeated at 2-3-week intervals in the presence of stable disease until proof of tumor progression; treatment was discontinued in the presence of progressive disease. Toxicity criteria were those of the Pediatric Oncology Group, and response criteria were those of the World Health Organization.

RESULTS

Treatment was well tolerated; only one patient developed leukopenia. One patient developed hyperpigmentation of dermal creases. Nausea and vomiting were mild to moderate. Six of the eight patients had no evidence of progression of disease while receiving BCD, and duration of stable disease activity for these six patients ranged from 1 to 5 months. Two other patients demonstrated progressive tumor enlargement while receiving the BCD combination.

DISCUSSION

The original trials with BCD justified its use on the supposition that it might contribute to a higher response rate (1). Previous studies by Rosen et al (2) indicated that a greater proportion of responses might be obtained with single agents or multiple agents when used early in the course of osteosarcoma rather than after prior treatment when drug resistance had been proven (3). In our patients who had received significant prior treatment, stable disease activity was obtained, yet no tumor regression could be measured for this group of individuals. Whether stable disease in these patients represented an antitumor effect or the natural history of osteosarcoma could not be determined. With none of the eight patients showing regression of tumor, one could not reject a hypothesis that the efficacy rate is as high as 37% using a two-sided significance level of 0.05. A 95% confidence interval for the patients with stable disease is 35%–97%. Conversely, the 95% confidence interval for the patients with progressive disease is 3%–65%.

[1] Received Mar 3, 1986; revised Sept 2, 1986; accepted Dec 12, 1986.

[2] Supported by Public Health Service grants CA-23099, CA-03713, and CA-21965 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by the American Lebanese Syrian Associated Charities. S. Epelman is the recipient of a traveling fellowship grant from the Pan American Health Organization.

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TABLE 1.—Charact

Patient No.	Age (yrs, mos)	Race & sex*	Primary tumor site; treatment	Duration o disease prio BCD treatm (mos)
1	12,4	WF	Left femur; amputation	37
2	16,1	WF	Multifocal (largest right femur); no extirpation	7
3	12,4	BF	Right ilium; unresected	6
4	13,4	WM	Right femur; amputation	6
5	9,8	WF	Multifocal; no extirpation	5
6	9,1	WM	Right humerus; Tikhoff-Lindberg	13
7	14	WM	Left femur; disarticulation	46
8	14	WM	Right humerus; disarticulation	4

*W = white; B = black; M = male; F = female.

†MTX-CF = methotrexate with leucovorin; CY = cyclophosphamide; DOX = MEL = melphalan; XRT = irradiation.

‡SD = stable disease; PD = progressive disease.

haracteristics of patients and results of treatment with BCD

ation of e prior to treatment mos)	Prior therapy†	Performance status (ECOG)	Sites of disease evaluated after BCD	No. of BCD courses	Result‡
37	MTX-CF; CY-DOX; DDP, VP-16, BIS	1	Maxilla	3	SD (2 mos)
7	DDP-DOX; MTX-CF; MIT	2	Multiple; bones, lungs, neck nodes	2	SD (1 mo)
6	DDP-DOX; MTX-CF	1	Ilium, pelvis, and sacrum	2	PD
6	MTX-CF; CY, DOX	1	Both lungs	1	PD
5	DDP, DOX; MTX-CF	2	Both lungs and bones	5	SD (5 mos)
13	DDP-DOX; MTX-CF	2	Lung	3	SD
46	DOX, VIN, MTX-CF; MEL, XRT lungs	2	Lung	3	SD (1 mo)
4	MTX-CF; VIN; XRT lungs	2	Lung	3	SD (3 mos)

DOX = doxorubicin; DDP = cisplatin; VP-16 = etoposide; BIS = bisantrene; MIT = mitoxantrone; VIN = vincristine;

Our results do not support the use of BCD for overt disease in patients with osteosarcoma who have received prior chemotherapy with multiple agents.

REFERENCES

1. MOSENDE C, GUTIERREZ M, CAPARROS B, ET AL. Combination chemotherapy with bleomycin, cyclophosphamide and dactinomycin for the treatment of osteogenic sarcoma. Cancer 40:2779–2786, 1977.
2. ROSEN G, CAPARROS B, HUVOS AG, ET AL. Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. Cancer 49:1221–1230, 1982.
3. PRATT CB. The treatment of metastatic osteogenic sarcoma. *In* PharManual: A Comprehensive Guide to the Therapeutic Use of Methotrexate in Osteogenic Sarcoma (Rosen G, ed). Morristown, NJ, PharmaLibri, 1984, pp 31–46.

Phase II Trial of Tiazofurin in Recurrent Squamous Cell Carcinoma of the Head and Neck[1,2]

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Tiazofurin, a C-nucleoside compound structurally related to the antiviral agent ribavirin (1), has shown activity against P388 leukemia, L1210 leukemia, and Lewis lung carcinoma cell lines (2). Its mechanism of action has been extensively evaluated in vitro and in vivo (3,4). Because of this preclinical antitumor activity, clinical trials were initiated.

We present results of a phase II trial of tiazofurin in recurrent squamous cell carcinoma of the head and neck previously untreated with chemotherapy.

PATIENTS AND METHODS

Fourteen patients with recurrent squamous cell carcinoma were treated. Eligible patients had a life expectancy $\geq$ 12 weeks, Zubrod performance status $\leq$ 2, and measurable disease. Absolute granulocyte count $>$ 2000/mm^3, platelet count $>$ 100,000/mm^3, total bilirubin $<$ 1.5 mg/dl, and serum uric acid $\leq$ 8.0 mg/dl were required.

The initial dose of 800 mg/m^2 was infused iv over 30 minutes daily for 5 consecutive days and repeated every 21 days. Allopurinol (300 mg/day) was begun prior to chemotherapy and continued for 6 consecutive days. With subsequent courses, the dose was escalated by 50%, except as follows. With grade 1 (mild) toxicity, the dose was increased by 25%; with grade 2 (moderate) toxicity, the dose remained unchanged; and with grade 3 (severe) toxicity, the dose was decreased by 25%.

Evaluation of response followed the standard criteria.

RESULTS

Patient characteristics and toxicity are shown in tables 1 and 2. One patient did not have biopsy-proven tumor and represented a protocol violation. Four patients received one course and one patient received two courses of treatment each at 800 mg/m^2. Six patients each received two courses with dose escalation to 1200 mg/m^2; two patients each received four courses escalating up to 1200 mg/m^2 and 1400 mg/m^2 with one patient escalated to 1000 mg/m^2. No objective responses were seen with this treatment; in 13 evaluable patients, a 21% response rate can be rejected at the $P = 0.05$ level of significance.

TABLE 1.—Patient characteristics

Characteristics	No. of patients (%)
Total evaluable	
For response	13
For toxicity	14
Median age in yrs (range): 60.5 (37-80)	
Male:female	13:1
Median Zubrod performance status (range): 1 (0-2)	
Number of courses: 28	
Primary site of disease	
Oral cavity	4
Oropharynx	3
Hypopharynx	2
Nasopharynx	2
Larynx	2
Unknown primary	1
Prior treatment	
Surgery and radiotherapy	9 (64)
Radiotherapy	5 (36)
Site of recurrent disease	
Primary site	4 (29)
Regional nodal site	2 (14)
Primary and regional nodal site	2 (14)
Distant metastases	4 (29)
Locoregional and distant	2 (14)

[1] Received May 12, 1986; revised Nov 18, 1986; accepted Dec 12, 1986.
[2] Supported by contract N01-CM-57739 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health and Human Services.
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TABLE 2.—Drug toxicity

Toxic effects	No. of patients (%)
Nausea	4 (28)
Vomiting	5 (36)
Diarrhea	1 (7)
Stomatitis	2 (14)
Myalgia/arthralgia	7 (50)
Diffuse weakness	1 (7)
Renal*	2 (14)
Exfoliative dermatitis	1 (7)
Conjunctivitis	3 (21)
Pericarditis†	1 (7)
Central nervous system‡	6 (43)
Serum enzyme elevation (mean)§	
CPK (1185 mU/ml)	14 (100)
SGOT (126 mU/ml)	4 (28)
LDH (340 mU/ml)	8 (57)

*Serum creatinine, 1.8 mg/dl.
†Reversible (at a dose of 1200 mg/m^2).
‡Includes: confusion (1 episode at 800 mg/m^2), headache (5 episodes).
§CPK = creatine phosphokinase; SGOT = glutamic oxaloacetic transaminase; and LDH = lactate dehydrogenase.

At 1000 mg/m^2, one patient had a granulocyte count of 1700 cells/mm^3 and one patient had a nadir platelet count of 55,000 cells/mm^3. At 1200 mg/m^2, two patients had nadir platelet counts of 72,000 and 75,000 cells/mm^3. One patient was stepwise escalated to 1400 mg/m^2 and, with a granulocyte count of 50 cells/mm^3, developed fever and pneumonia.

DISCUSSION

Because of the consistently poor survival rate in patients with recurrent head and neck cancer, the search for newer agents with potential activity is imperative. Our ability to adequately evaluate these drugs is maximized in patients unexposed to conventional cytotoxic agents. Tiazofurin has nevertheless shown no activity, despite use in an unlikely environment for drug resistance.

Toxic side effects were mild and self-limited.

This phase II trial yielded no evidence of antitumor activity, but because of the potential for severe and unpredictable intolerable toxicity (5), further evaluation at doses > 1400 mg/m^2/day $\times$ 5 and in patients with prior therapy should be undertaken with caution.

REFERENCES

1. WITKOWSKI JT, ROBINS RK, SIDWELL RW, ET AL. Design, synthesis, and broad-spectrum antiviral activity of 1-β-D-ribo-furanosyl-1,2,4-triazole-3-carboxamide and related nucleosides. J Med Chem 15:1150–1154, 1972.
2. ROBINS RK, SRIVASTAVA PC, NARAYANAN VL, ET AL. 2-β-D-Ribofuranosylthiazole-4-carboxamide, a novel potential antitumor agent for lung tumors and metastases. J Med Chem 25:107–108, 1982.
3. COONEY DA, JAYARAM HN, GEBEYEHU G, ET AL. The conversion of 2-β-D-ribofuranosylthiazole-4-carboxamide to an analogue of NAD with potent IMP dehydrogenase-inhibitory properties. Biochem Pharmacol 31:2133–2136, 1982.
4. JAYARAM HN, COONEY DA, GLAZER RI, ET AL. Mechanism of resistance to the oncolytic C-nucleoside 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC-286193). Biochem Pharmacol 31:2557–2560, 1982.
5. TRUMP DL, TUTSCH KD, KOELLER JM, ET AL. Phase I clinical study with pharmacokinetic analysis of 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC-286193) administered as a five-day infusion. Cancer Res 45:2853–2858, 1985.

Esorubicin in Refractory Metastatic Carcinoma of the Breast: A Northern California Oncology Group Study[1,2]

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Esorubicin (4′-deoxydoxorubicin) is an anthracycline analogue that has demonstrated activity in preclinical systems and minimal cardiac toxicity (1-3). We report here a Northern California Oncology Group (NCOG) phase II study of esorubicin in patients with metastatic breast cancer.

METHODS

Eligible patients had histologically confirmed breast cancer refractory to non-anthracycline-containing chemotherapy and to hormonal therapy if estrogen receptor status was positive or unknown. They had to have adequate hepatic, renal, and bone marrow function; a Karnofsky performance status $\geq 50\%$; an estimated survival ≥ 8 weeks; and a physiologic age ≤ 70 years. Patients were ineligible if they had received prior anthracycline therapy or if they had a history of significant cardiac disease, concurrent complications such as infection, active second tumor, or central nervous system metastasis. All patients gave signed informed consent.

The initial five patients were treated with esorubicin at a dose of 35 mg/m^2 iv every 3 weeks. After our initial experience revealed greater myelosuppression than anticipated, the starting dose was decreased to 30 mg/m^2. Subsequent doses of esorubicin were modified based upon day-of-treatment blood cell counts, according to the following criteria: wbcs $\geq$ 3500 and platelets $\geq$ 150,000/mm^3—100% dose; wbcs = 3000-3499 or platelets = 100,000-149,999/mm^3—75% dose; wbcs $<$ 3000 or platelets $<$ 100,000/mm^3—delay of 1 week and recheck of blood cell counts.

Toxicity was assessed by NCOG toxicity criteria.

TABLE 1.—Patient characteristics

Characteristic	No. of patients (%)
Total	27
Evaluable	26 (96)
Ineligible	1 (4)
Mean age in yrs (range): 56 (28-74)	
Median Karnofsky performance status (range): 80 (60-100)	
Site of disease	
Local-regional	13 (50)
Bone	16 (62)
Lung	14 (54)
Liver	7 (27)
Prior therapy	
Mastectomy	20 (77)
Radiotherapy	19 (73)
Chemotherapy*	26 (100)
CMF	21 (81)
CMFV	2 (8)
CMFVP	2 (8)
PF	2 (8)
Hormonal therapy	19 (73)
Estrogen receptor status	
Positive	10 (39)
Negative	12 (46)
Unknown	4 (15)
Progesterone receptor status	
Positive	3 (12)
Negative	6 (23)
Unknown	17 (65)

*CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; CMFV = CMF plus vincristine; CMFVP = CMFV plus prednisone; and PF = melphalan and 5-fluorouracil.

[1] Received Sept 2, 1986; revised Dec 8, 1986; accepted Dec 18, 1986.
[2] Supported by Public Health Service grants CA-21744, CA-39098, CA-19408, CA-35014, CA-33572, and CA-25862 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by a grant from Farmitalia Carlo Erba.
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TABLE 2.—Drug toxicity

Toxic effect	No. of patients (%)
Wbc count nadir (cells/mm^3)	
3000–4000	2 (8)
2000–2999	6 (23)
1000–1999	8 (31)
< 1000	8 (31)
Platelet count nadir (cells/mm^3)	
90,000–129,000	1 (4)
50,000–89,000	2 (8)
25,000–49,000	5 (19)
< 25,000	3 (12)
Fever > 38°C	19 (73)
Nausea and vomiting (grade)	
Minimal (1)	6 (23)
Moderate (2)	6 (23)
Severe (3–4)	3 (12)
Mucositis (grade)	
Minimal (1)	2 (8)
Moderate (2)	4 (15)
Severe (3)	1 (4)
Cutaneous erythema or wheal and flare reaction	3 (12)

Cardiac toxicity was assessed by clinical examination, by radionuclide cardiac ejection fractions, and by the endomyocardial biopsy technique (4).

RESULTS

Twenty-seven patients were registered to study, and 26 patients were eligible and evaluable. One patient was ineligible because of prior therapy with doxorubicin. The characteristics of the 26 evaluable patients are shown in table 1.

Toxicity is shown in table 2. One of our patients treated with a single esorubicin dose of 35 mg/m^2 experienced profound pancytopenia lasting > 5 weeks. No patient experienced clinical cardiac toxicity from drug, although pathologic changes consistent with anthracycline injury were documented by endomyocardial biopsy.

A median of 2.5 courses of esorubicin were administered, with a mean cumulative dose of 76 mg/m^2 (range, 30–262). One complete response was observed in skin, one partial response in lymph nodes, and one partial response in lung. Median survival was 9 months from the onset of treatment.

The patients treated in this phase II trial of esorubicin were heavily pretreated and had a high rate of visceral disease. Our results document a low rate of antitumor response of 12% (95% confidence interval of 0–24%) at esorubicin doses of 30–35 mg/m^2 every 3 weeks. Toxicity in our patients was significant. Leitner et al (5) have studied esorubicin at a lower dose of 25 mg/m^2 every 3 weeks and observed no responses in 20 patients. We conclude that there is minimal activity and substantial toxicity when esorubicin is administered at these doses to women with breast carcinoma who have previously failed combination chemotherapy.

REFERENCES

1. BELLINI O, SAVI G, and CASAZZA AM. Investigation on the antitumor activity and cardiotoxicity of anthracyclines in C3H mice. *In* Proceedings of the 12th International Congress of Chemotherapy, Florence, Italy. The International Society of Chemotherapy, 1981, p 301.
2. CASAZZA AM, SAVI G, PRATESI G, ET AL. Antitumor activity of 4′-deoxydoxorubicin in mice. *In* Current Chemotherapy and Immunotherapy (Periti P, and Gross GG, eds). Washington, DC, American Society for Microbiology, 1982, pp 1433–1435.
3. SALMON SE, YOUNG L, SOEHNLEN B, ET AL. Antitumor activity of esorubicin in human tumor clonogenic assay with comparisons to doxorubicin. J Clin Oncol 2:282–286, 1984.
4. BRISTOW MR, LOPEZ MB, MASON JW, ET AL. Efficacy and cost of cardiac monitoring in patients receiving doxorubicin. Cancer 50: 32–41, 1982.
5. LEITNER SP, CASPER ES, HAKES TB, ET AL. Phase II trial of 4′-deoxydoxorubicin in patients with advanced breast cancer. Cancer Treat Rep 69:1319–1320, 1985.

Phase II Study of the Combination of Carmustine and 6-Thioguanine in Advanced Malignant Melanoma[1,2]

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For the North Central Cancer Treatment Group

Durable palliation for patients with disseminated malignant melanoma remains an elusive goal. Conventional cytotoxic and recombinant interferon programs have demonstrated some, but limited, efficacy (1,2). Alternative systemic strategies are appropriate for clinical trials in this neoplasm.

Carmustine (BCNU), a chloroethyl-substituted nitrosourea, has activity against malignant melanoma (3). A typical iv dose is 200 mg/m^2 every 6 weeks in previously untreated patients.

6-Thioguanine (6-TG), an antimetabolite which impairs purine metabolism, produces acceptable myelosuppression and little gastrointestinal toxicity from an iv dose of 700 mg/m^2 every 21 days (4). The agent is therapeutically synergistic with all major classes of alkylating agents (5). Simultaneous treatment of advanced L1210 leukemia with a nitrosourea plus 6-TG resulted in high cure rates. In light of these observations, the Mayo Clinic conducted a pilot study of BCNU plus 6-TG in 21 patients with a spectrum of advanced cancers. An appropriate dosage regimen based on those patients was 250 mg/m^2 of BCNU with 700 mg/m^2 of 6-TG administered every 6 weeks.

MATERIALS AND METHODS

Thirty-five patients with histologically confirmed, measurable disseminated malignant melanoma participated in the clinical trial after providing written informed consent (table 1). Each patient had adequate hematologic (wbc count $> 4000/mm^3$; platelet count $> 130,000/mm^3$), renal (serum creatinine < 1.5 mg/dl), and hepatic (no elevation of direct-reacting serum bilirubin) parameters.

Objective response was defined as a $\geq 50\%$ decrease in the sum of the products of the longest perpendicular

TABLE 1.—Patient characteristics

Characteristic	No. of patients
Total	35
Median age in yrs (range): 54 (26–74)	
Sex	
Male	21
Female	14
ECOG PS*	
0	14
1	13
2, 3	8
Previous chemotherapy	
Yes	10
No	25
Dominant metastasis	
Visceral	23
Nonvisceral	12

*Eastern Cooperative Oncology Group PS: 0 (fully active) to 4 (totally disabled).

[1] Received Sept 4, 1986; revised Dec 8, 1986; accepted Jan 5, 1987.

[2] Supported by Public Health Service grant CA-25224 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

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TABLE 2.—Toxicity

Toxic effect	
No. of patients	
Hematologic	
Wbc count nadir (cells/mm^3)*	
< 1000	2
1000-3000	14
Platelet count nadir (cells/mm^3)†	
< 25,000	3
25,000-100,000	13
% of patients	
Gastrointestinal	
Nausea	
Any	89
Moderate-severe	66
Vomiting	
Any	80
Moderate-severe	60
Anorexia	
Any	23
Moderate-severe	20

*Median, 3.25×10^3 cells/mm^3; range, $0.8–1.6 \times 10^3$ cells/mm^3.
†Median, 105×10^3 cells/mm^3; range, $10–268 \times 10^3$ cells/mm^3.

diameters of the most clearly measurable indicator without new lesions or symptomatic deterioration. Progressive disease was defined as a > 25% increase in any indicator lesion or the appearance of new lesions.

RESULTS

Therapeutic.—Among 20 "good-risk" patients [performance scale (PS) 0 or 1, or no prior chemotherapy] we documented two partial regressions of soft tissue lesions for 9 and 16 months. Among 15 "poor-risk" patients (PS 2 or 3, or prior chemotherapy) there were four partial regressions (soft tissue, 3, 3, 6, and 9 months). Overall, the median times to disease progression and survival were 1.4 and 5 months, respectively.

Toxicity.—Toxic effects, generally transient and reversible, were hematologic and gastrointestinal (table 2).

DISCUSSION

We achieved an objective response rate of 17% from BCNU plus 6-TG with reasonably acceptable toxic effects. A 95% confidence interval for the probability of response to this combination is 0.006, 0.33. With noteworthy exceptions, the responses observed were transient. It is exceedingly unlikely that this two-agent regimen will significantly influence the currently inexorable course of most patients with disseminated malignant melanoma.

REFERENCES

1. LUIKART SD, KENNEALEY GT, and KIRKWOOD JM. Randomized phase II trial of vinblastine, bleomycin, and *cis*-dichlorodiammine-platinum(II) versus dacarbazine in malignant melanoma. J Clin Oncol 2:164-168, 1984.
2. CREAGAN ET, AHMANN DL, FRYTAK S, ET AL. Phase II trials of recombinant leukocyte A interferon in disseminated malignant melanoma: results in 96 patients. Cancer Treat Rep 70:619-624, 1986.
3. RAMIREZ G, WILSON W, GRAGE T, ET AL. Phase II evaluation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU: NSC-409962) in patients with solid tumors. Cancer Chemother Rep 56:787-790, 1972.
4. DENES A, and PRESANT C. 6-Thioguanine (TG): a phase I study of intermittent oral (po) and intravenous (iv) therapy in solid tumors. Proc Am Assoc Cancer Res and ASCO 20:107, 1979.
5. FUJIMOTO S, ANAGAKI J, HORIKOSHI N, ET AL. Combination chemotherapy of 6-thioguanine with various antitumor agents against murine L-1210 leukemia. Gann 68:543-552, 1977.

"Ifosfamide Plus *N*-Acetylcysteine in the Treatment of Small Cell and Non-Small Cell Carcinoma of the Lung: A Southeastern Cancer Study Group Trial"

This article reports on 84 evaluable patients with lung cancer treated with ifosfamide and *N*-acetylcysteine (1). It concluded that this drug does not give valuable results in lung cancer. Ifosfamide had been proposed in the past for the management of non-small cell lung cancer, but was rejected by the Food and Drug Administration on the advice of Dr. Einhorn, one of the authors of this article. His reasons were as follows: (*a*) the results of the study supporting the proposal (2) were too good to be acceptable; and (*b*) there was no randomized study comparing a treated group with a control group or with a cyclophosphamide-treated group. In the present study, the Southeastern Cancer Study Group had a chance to answer these criticisms. However, they gave the drug with *N*-acetylcysteine, which had not been done in the earlier study (2). The drug was given at a dose which produced minimal myelosuppression. They also chose to do a single-arm study instead of a randomized study. Even so, they achieved three partial remissions among 25 patients (12%) with non-small cell lung cancer who had had prior exposure to therapy, which is better than the response in untreated patients (two responses among 41 patients)! It is also interesting to note that a single agent achieved a median survival of 23.6 weeks in patients with non-small cell carcinoma. This should be compared with the results reported by Bodey et al (3), who compared cyclophosphamide alone in a randomized study versus cyclophosphamide, vincristine, semustine, and bleomycin (COMB), a regimen reported earlier by Livingston et al (4). The median survival for COMB was only 10 weeks; this was inferior to cyclophosphamide alone, where the median survival was 19.8 weeks. The Southeastern Oncology Group has been able to demonstrate in this study that this agent is active. Their conclusion, "that further trials with ifosfamide in bronchogenic carcinoma are not warranted," does not seem justified.

REFERENCES

1. LOEHRER PJ, SR, BIRCH R, KRAMER BS, ET AL. Ifosfamide plus *N*-acetylcysteine in the treatment of small cell and non-small cell carcinoma of the lung: a Southeastern Cancer Study Group trial. Cancer Treat Rep 70:919–920, 1986.
2. COSTANZI JJ, MORGAN LR, and HOKANSON J. Ifosfamide in the treatment of extensive non-oat cell carcinoma of the lung. Semin Oncol 9(suppl 1):61–65, 1982.
3. BODEY GP, LAGAKOS SW, GUTIERREZ AC, ET AL. Therapy of advanced squamous carcinoma of the lung. Cyclophosphamide versus "COMB." Cancer 39:1026–1031, 1977.
4. LIVINGSTON RB, EINHORN LH, BODEY GP, ET AL. COMB (cyclophosphamide, vincristine, methyl-CCNU and bleomycin): a four-drug combination in solid tumors. Cancer 36:327–332, 1975.

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August 27, 1986

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Reply

We wish to thank Dr. Holoye for reading our brief article as well as the opportunity to answer his comments regarding the scientific merit of our paper. We do not feel, however, that this is the forum for debate regarding the proceedings of the Food and Drug Administration Oncology Advisory Committee but suffice it to say that the decision to withhold the release of ifosfamide for the indication of non-small cell lung cancer was not a unilateral but a broad-based decision. The remainder of his criticisms will be attempted to be answered individually.

N-Acetylcysteine was used with ifosfamide as a uroepithelial protective agent. While it is true that this drug was not used in the earlier studies by Costanzi et al (1) and Morgan et al (2), Holoye et al (3) reported that "an improved therapeutic ratio of ifosfamide could be attained by combining (with) the therapeutically inactive agent, acetylcysteine. . . ." Furthermore, in another paper Holoye et al (4) also stated that "ifosfamide and other oxyphosphorines can be given simultaneously with a NAC without abolishing the tumor activity of the first drug."

While it is true that the myelosuppression in our report was relatively mild, central nervous system tox-

icity occurred in a quarter of the patients while a third had moderate to severe weight loss related to this therapy. Regardless, the purpose of this study was to confirm the positive results of other investigators by using the identical dose regimen.

Dr. Holoye also expressed a preference towards a randomized trial of ifosfamide in place of this single-arm study. An unpublished randomized trial had been performed by the Southwestern Oncology Group (R. Livingston, personal communication) in which patients were randomized to receive ifosfamide alone versus ifosfamide plus doxorubicin versus ifosfamide plus doxorubicin plus lomustine, with response rates of 12%, 7%, and 13%, respectively. This study, however, was criticized (prior to publication) because of a lower ifosfamide dose (1.0 g/m^2 on Days 1-5). We therefore felt that a phase II trial evaluating response rate and overall survival would be sufficient to confirm the activity of ifosfamide while minimizing the number of patients required to complete the study.

While we are delighted that Dr. Holoye has discovered the means by which one can turn our negative trial into a positive one, we are reluctant to wholeheartedly accept his rationale. We disagree that our trial should be compared with the results reported by Bodey et al (5) for a variety of reasons. In that study of COMB (cyclophosphamide, vincristine, semustine, and bleomycin) versus cyclophosphamide, 34% of the 64 randomized patients were *not ambulatory* at the time of placement in the study and 17 patients were not evaluable. Only two patients responded, one in each arm. Overall, the median survival time was 14.4 weeks for cyclophosphamide versus 9 weeks for the COMB arm, which was not significant although there was borderline significance ($P = 0.05$) when one looked at the remaining 31 evaluable ambulatory patients. It is not clear as to whether Dr. Holoye feels that vincristine, methotrextrate, and bleomycin detract from the "activity" of cyclophosphamide.

Similarly, the "improved" median survival time in our study is misleading. For example, the BACON (bleomycin, doxorubicin, lomustine, vincristine, and mechlorethamine) regimen has also been frequently used as a useful historical "control" (6). Like the COMB study, 20% of the patients had a Karnofsky performance status < 50; despite this, the median survival time was 20 weeks. Additionally, Pannuti et al (7) compared cyclophosphamide alone to cyclophosphamide, methotrextrate, 5-fluorouracil, and hydroxyurea and found only one response among 29 patients treated with cyclophosphamide. The median survival time for this group was 24 weeks. At Indiana University a randomized trial comparing cyclophosphamide alone or with doxorubicin plus methotrexate demonstrated no differences in the median survival times (32 vs 25 weeks, respectively) (8). Furthermore, a randomized trial comparing cisplatin plus vindesine versus supportive care alone was presented by Dr. Woods at the American Society of Clinical Oncology. Although 30% of the patients responded to the combination chemotherapy there was no difference in the median survival time of 22 weeks for all patients (24 weeks for chemotherapy patients; 21 weeks for controls). Thus, the median survival time of our patients treated with ifosfamide is quite comparable to that seen with supportive care alone or ineffective single agents in the 1980s.

If one wishes to devote research efforts in non-small cell lung cancer using agents producing a $< 10\%$ response rate and median survival times comparable to supportive care alone, then we would agree with Dr. Holoye that further trials "might be justified." However, such trials are unlikely to make a major therapeutic impact in the treatment of this disease.

REFERENCES

1. COSTANZI JJ, MORGAN LR, and HOKANSON J. Ifosfamide in the treatment of extensive non-oat cell carcinoma of the lung. Semin Oncol 9:61-65, 1982.
2. MORGAN LR, POSEY LE, RAINEY J, ET AL. Ifosfamide: a weekly dose fractionated schedule in bronchogenic carcinoma. Cancer Treat Rep 65:693-695, 1981.
3. HOLOYE PY, ANDERSON T, DUELGE J, ET AL. Use and safety of high dose ifosfamide. Semin Oncol 9:78-86, 1982.
4. HOLOYE PY, DUELGE J, HANSEN RM, ET AL. Prophylaxis of ifosfamide toxicity with oral acetylcysteine. Semin Oncol 10:66-71, 1983.
5. BODEY GP, LAGAROS SW, GUTIERREZ AC, ET AL. Therapy of advanced squamous carcinoma of the lung: cyclophosphamide versus "COMB." Cancer 39:1026-1031, 1977.
6. LIVINGSTON RB, FEE WH, EINHORN LH, ET AL. BACON (bleomycin, Adriamycin, CCNU, Oncovin, and nitrogen mustard) in squamous cell lung cancer. Cancer 37:1237-1242, 1976.
7. PANNUTI L, LELLI G, CASADIO M, ET AL. High-dose cyclophosphamide versus cyclophosphamide, methotrexate, 5-FU, and hydroxyurea (CMFH) in the treatment of stage III non-small cell bronchogenic carcinoma: a randomized trial. Cancer Treat Rep 64: 1131-1134, 1980.
8. EINHORN LH, WILLIAMS SD, STEVENS EE, ET AL. Random prospective study of cyclophosphamide, doxorubicin, and methotrexate (CAM) combination chemotherapy versus single-agent sequential chemotherapy in non-small cell lung cancer. Cancer Treat Rep 66: 2005-2011, 1982.
9. WOODS RL, LEVI JA, PAGE J, ET AL. Non small cell cancer: a randomized comparison of chemotherapy with no chemotherapy. Proc ASCO 4:177, 1985.

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"Pharmacokinetics and Immediate Effects of High-Dose Carmustine in Man"

With respect to the paper by Henner et al (1), I was puzzled at the description of the reactions associated with the infusion of high doses of carmustine in humans, since others have not described such reactions (2).

In autologous experiments which we performed, 600 mg/m^2 of carmustine (BCNU) was given over a period of 30 minutes without significant reaction at all (unpublished data).

The method used to prepare the BCNU by many groups investigating high-dose regimens has involved dissolving all of the BCNU in as little alcohol as possible, rather than following the instructions of the package insert as did Henner and associates. It would be intriguing to see whether these acute reactions are ameliorated by preparing the BCNU infusion in this manner, thus avoiding the effects of acute alcohol infusion.

REFERENCES

1. HENNER WD, PETERS WP, EDER JP, ET AL. Pharmacokinetics and immediate effects of high-dose carmustine in man. Cancer Treat Rep 70:877-880, 1986.
2. PHILLIPS GL, FAY JW, HERTZIG GP, ET AL. Intensive 1,3-bis (2-chlorethyl)-1-nitrosourea (BCNU), NSC-436650 and cryopreserved autologous marrow transplantation for refractory cancer: a phase I–II study. Cancer 52:1792-1802, 1980.

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Unusual Skin Reaction to Echinomycin

Echinomycin is a cyclic peptide (a quinoxaline antibiotic) isolated from *Streptomyces echinatus* (1). This drug is currently undergoing phase II clinical trials for the treatment of multiple myeloma (MM), renal carcinoma, and non-Hodgkin lymphomas in our institution using an intermittent schedule (2). We report an unusual localized skin reaction from echinomycin that occurred in a patient with MM.

CASE REPORT

A 67-year-old black male with IgG MM was entered in the echinomycin protocol after experiencing disease progression on melphalan and prednisone. Echinomycin at a dose of 2225 μg (1250 μg/m^2) was given iv in 500 ml of normal saline over 1 hour, repeated every 28 days. The initial dose was given in a large vein in the right forearm without difficulty or symptomatic side effects. The second dose (Day 28) was also given through a large vein in the right forearm without difficulty. Specifically, there was adequate flow of the drug through the vein with no evidence of infiltration, no local discomfort, and no local erythema. Two days following this infusion the patient noted mild pruritis in the inner aspect of the right forearm beginning over the area the drug was infused. Five days after the infusion he noted a mild erythema and scaling of the skin on the medical aspect of the forearm extending from the wrist to the antecubital fossa. All of the above symptoms were sufficiently mild that he did not report them to his physician. The skin changes were noted by the physician when the patient reported 7 days after treatment for routine laboratory studies. The skin was intact, nonerythematous, of normal temperature, and nontender. An irregular, patchy, darkly pigmented discoloration was noted in a vascular distribution over the entire inner aspect of the forearm. Varying degrees of ulceration were seen along a vascular distribution, with some desquamation around the larger ulcers. The discoloration and ulcerations occurred proximal and distal to the injection site. The largest ulceration was distal to the injection site. Two weeks later, the ulcerative areas were healing well. Complete resolution of the ulcerative areas occurred within 4 weeks associated with lessening, but persistence, of skin discoloration in a vascular pattern. The third dose of echinomycin was given on schedule without difficulty in the left forearm. However, when the second dose was given in the left forearm (fourth total dose), skin changes similar to but much less severe than those previously described occurred.

COMMENTS

The mechanisms of the reaction we describe are unknown. This toxicity of echinomycin appears to be vasculocutaneous in nature. The physical findings follow the vascular pattern of the arm and a prior sensitizing exposure to the drug appears to be required since the reaction occurred after the second injection in each arm. Because the symptoms were mild and the patient did not report them promptly, there was no opportunity to determine if steroids or other forms of therapy would have obviated the full development of the observed reaction.

Nausea, vomiting, and occasionally mild phlebitis have been reported as common toxic effects of echinomycin. Allergic reactions have also occurred with this drug (3). These have included anaphylactoid reactions and are thought to be associated with the non-ionic surfactant cremophor, which is used as a vehicle for the

formulation of echinomycin. Reactions such as we have described have not been previously reported with echinomycin. Further observations of this phenomenon will be required to define the mechanisms of this reaction and what, if any, therapy can prevent or treat this self-limited side effect.

REFERENCES

1. CHABNER BA, and MYERS CE. Clinical pharmacology of cancer chemotherapy. *In* Cancer: Principles and Practice of Oncology (DeVita VT, Hellman S, and Rosenberg SA, eds), Philadelphia, Lippincott, 1985, p 291.
2. ANDREWS W, HARVEY J, MCFADDEN M, ET AL. A phase I study of echinomycin NSC #526417, administered on an intermittent bolus schedule. Proc ASCO 4:42, 1985.
3. LASSUS M, SCOTT O, and LEYLAND-JONES B. Allergic reactions associated with cremophor containing antineoplastics. Proc ASCO 4:268, 1985.

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Some Thoughts on the Future of Clinical Trials Groups in Cancer

We are a behavioral science research unit with a particular focus on physician participation in randomized clinical trials (1-4). We read with great interest the article regarding clinical trials that appeared in the February 1986 issue of *Cancer Treatment Reports* (5) and the responses published in September 1986 (6-8). Dr. Wittes and the respondents provide valuable insights on the potential future of clinical trials in cancer, from a variety of points of view. However, as scientists interested in the dynamics of group behavior, we believe there is a yet unexplored area of research which could provide an additional perspective on this complex issue.

We suggest that cooperative groups provide often unrecognized services to the enhancement of quality scientific research. For example, published data from the NSABP (1) as well as the findings of a 6-year survey of 484 oncologists (9,10) indicate that physician willingness to enter eligible patients onto protocol is strongly influenced by the cooperative group mechanism. This is particularly true for those physicians who feel they "work in isolation" among colleagues who do not share their research interests. The opportunity to meet regularly with others interested in the promotion of clinical trials was cited as a major incentive for physicians to place patients on study. Whether increasing direct funding to individual institutions while decreasing the impact of cooperative group influence will ensure and improve ongoing physician participation in clinical trials is a significant question. Second, the concept of cooperative groups provides an opportunity for less personal peer-reviewed quality control. This appears to be of importance to some physicians who are willing to submit their records to group audit, but are less willing to be reviewed by direct colleagues. Third, physicians have been trained to act as individual decision-makers and are often reluctant to adhere to prescribed treatment options. The pooling of standardized patient information has not been an established pattern of traditional medical practice. Cooperative groups are thus obliged to maintain high standards of data control from physicians who may be both unfamiliar and uncomfortable with the need for such rigor. Maintaining the uniform standards without a "direct governing body" may be difficult.

Finally, the ability of cooperative groups and those with the particular good fortune to have a charismatic leader to initiate and sustain physician enthusiasm cannot be overlooked. Many of the physicians we studied believe that they are personally responsible for maintaining the interest and participation of their colleagues. They explain that they themselves need to be regularly regenerated to perform this often time- and energy-consuming task. Without regular injections of support which they found at the meetings, they felt it would be difficult to sustain their motivation. In addition, the opportunity to provide input and receive feedback regarding the actual design and implementation of trials is also felt to be important. They believe that the cross-fertilization of shared experiences, both formally and informally presented, is of great importance. Physicians agreed that sharing their concerns regarding trial management with institutional colleagues, already skeptical about the clinical trial process, was often frustrating and unproductive.

These comments have not been designed to endorse or promote the role of cooperative groups. Rather, we hope that this will provide food for thought for those reviewers who are attempting to assess the effectiveness of the clinical trial process. Since Dr. Wittes clearly states that "future efforts to expand patient and physician participation in trials will probably be limited by a fixed pool of funds," we suggest that a sociological review of the benefits and drawbacks of the cooperative group mechanism may provide particularly timely and ultimately cost-effective information. Specifically, a comprehensive and inclusive evaluation of the behavioral impact of the current cooperative group process may greatly facilitate the future introduction of effective intergroup research. Attempts to assess each group on a study-by-study basis may indeed result in a detailed

evaluation while missing a critically important function of the group as an effective mechanism for social control and enhancement of the clinical trial process.

REFERENCES

1. TAYLOR KM, MARGOLESE RG, and SOSKOLNE CL. Physicians' reasons for not entering eligible patients into a randomized clinical trial for breast cancer surgery. N Engl J Med 310:1263–1267, 1984.
2. TAYLOR KM. The doctor's dilemma: participating in randomized clinical trials. Cancer Treat Rep 69:1095–1097, 1985.
3. TAYLOR KM, and KELNER MJ. Informed consent: the physician's perspective. Soc Sci Med 24:2, 135–143, 1987.
4. TAYLOR KM. Telling bad news: physicians and the disclosure of undesirable information. *In* Sociology of Health and Illness. In press.
5. WITTES RE, FREIDMAN MA, and SIMON R. Some thoughts on the future of clinical trials in cancer. Cancer Treat Rep 70:241–250, 1986.
6. CRAWFORD ED. "Some thoughts on the future of clinical trials in cancer." Cancer Treat Rep 70:1133, 1986.
7. PATER JL, EISENHAUER E, SHELLEY W, ET AL. Testing hypotheses in clinical trials: experience of the National Cancer Institute of Canada Clinical Trials Group. Cancer Treat Rep 1133–1136, 1986.
8. HIGBY DJ. "Some thoughts on the future of clinical trials in cancer." Cancer Treat Rep 70:1136, 1986.
9. TAYLOR KM. Interpreting physician participation in randomized clinical trials—the Physician Orientation Profile, in review.
10. AGE OF WISDOM: Questioning Medical Tradition, Study Guide and Videotape, University of Toronto, 1985.

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BOOK REVIEW

Cancer Immunology: Innovative Approaches to Therapy. **R. B. Herberman, editor. Boston, Martinus Nijhoff Publishers, 1986, $55.00, 229 pp, ISBN 0-893838-757-4**

Interest in cancer immunology has ebbed and flowed really since the turn of the century. Early enthusiasm for the subject was quickly tempered by the appreciation that transplanted tumor rejections seen in experimental animals were not due to specific antitumor immune responses but rather to histocompatibility differences between different strains of the same laboratory animals. In the fifties, however, tumor immunologists utilizing newly developed inbred strains of experimental animals provided conclusive demonstration of tumor-specific or tumor-associated immune reactions against tumors produced by both viruses and chemicals. That spurred renewed and continued investigator activity which has led most recently to the development of biological response modifier therapy as a legitimate new modality of anticancer treatment. This slim volume published in the "Cancer Treatment and Research" series provides a collection of papers which reviews the basic and applied clinical investigative basis for this new form of treatment.

A surprisingly wide range of topics is covered quite effectively and even in some depth in a book which contains fewer than 250 pages. Robert Gallo, whose work with Interleukin-2 and AIDS (acquired immune deficiency syndrome) has contributed so much to our knowledge of the new field of biological response modifier therapy, is the senior author on a report which clearly and concisely covers the immunological aspects of HTLV infection, with a particular focus on seroepidemiological studies. Ronald Herberman, who is also the book's editor, summarizes his group's work and the work of others establishing a role for natural killer (NK) cells in the control of tumor growth and spread. He does a particularly good job with this and offers some new perspectives. A fine section by Pierre Henkart discusses the various means by which NK cell-mediated cytotoxicity occurs. Dolph Adams et al consider, in a balanced and thorough manner, the potentially important role of the macrophage in the destruction of tumor cells. The evidence for the macrophage's role in immune surveillance is presented clearly and in impressive detail.

The book concludes with two important chapters, one on monoclonal antibody therapy for cancer by Kenneth Foon and Charles Morgan and the other on possible Interleukin-2 applications in cancer therapy by Roland Mertlesmann and Karl Welte. Here one finds the major shortcoming of this type of edited review, and it is one which is perhaps unavoidable. The book was published in 1986. Although in most chapters a serious attempt was made to review material and to bring references up to 1984 and even 1985, the sections on monoclonal antibodies and Interleukin-2, although providing useful background information about the development of these areas, are already dated. Thus, Rosenberg's exciting new work exploring the toxicity and clinical effectiveness of Interleukin-2 alone or in combination with LAK cells is not covered, although the basic work leading to the clinical studies is reviewed. Similarly, the recent successful use of monoclonal antibodies in cutaneous T-cell lymphoma is not covered. Also, a survey of interferon therapy would have been more in place in a volume of this sort, rather than the weak chapter on tumor-specific antigens induced by mutagens and DNA-hypomethylating agents which is provided. This, though, is a question of editorial judgment, and by and large this work achieves its goal of providing a good summary of and an introduction to the new cancer immunology. The account is lively and illuminates admirably the studies which have brought us to our present promising circumstances.

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January 5, 1987

ANNOUNCEMENTS

NIH Centennial Symposia and Reunion

The National Institutes of Health will hold a reunion of its alumni in Bethesda, MD, on October 15–18, 1987, as a conclusion to its centennial year. All those who have worked or studied at NIH are invited. The weekend features 16 scientific symposia and a series of social events. Symposia on Thursday and Friday will be on campus; Saturday's plenary symposium on Frontiers in Biomedical Science will be held in the Kennedy Center Concert Hall. A banquet on Saturday night in the Pension Building and a Sunday brunch on campus conclude the weekend.

For detailed information, send a card with name and address to: Ms. Andrea Manning, NIH Centennial, c/o Prospect Associates, 1801 Rockville Pike, Rockville, MD 20852 (telephone: 301-468-6555).

International Conference on Reproduction and Human Cancer

Bethesda, Maryland, May 11–13, 1987

For further information, contact: Drs. John J. Mulvihill (Landow Bldg, Rm 8C41) or Richard J. Sherins (Bldg 10, Rm 10N234), National Institutes of Health, Bethesda, MD 20892; or call Conference Manager, Ms. Mary E. Clark (telephone: 301-589-6760).

Seventy-Eighth Annual Meeting of the American Association for Cancer Research

Atlanta, Georgia, May 20–23, 1987

For further information, contact: American Association for Cancer Research Office, Temple University School of Medicine, West Bldg, Rm 301, Philadelphia, PA 19140 (telephone: 215-221-4565).

Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis

Pittsburgh, Pennsylvania, June 8–12, 1987

For further information, contact: R. Hilda Diamond, Associate Director, Biomedical Engineering Program, Carnegie Mellon University, Pittsburgh, PA 15213-3890 (telephone: 412-268-2521).

Radiotherapy and Combined Treatments

Milan, Italy, June 15–17, 1987

For further information, contact: Miss Lucia Manfredi, Ufficio Attività Didattiche, Istituto Nazionale Tumori, Via G. Venezian 1, 20133 Milan, Italy (telephone: 02/2363764).

Third International Conference on Advances in Regional Cancer Therapy of the Association of Regional Tumor Therapy

Ulm, Federal Republic of Germany, September 9–12, 1987

For further information, contact: Conference Secretary, Mrs. G. Mutschler, Department of Oral and Maxillofacial Surgery, University of Ulm—Bundeswehrkrankenhaus, Oberer Eselsberg 40, PO Box 1220, D-7900 Ulm, Federal Republic of Germany (telephone: 07 31/176-3579).

Second International Head and Neck Oncology Research Conference

Arlington, Virginia, September 10–12, 1987

For further information, contact: Gregory T. Wolf, MD, Veterans Administration Medical Center, Clinical Studies Office (153), 2215 Fuller Rd, Ann Arbor, MI 48105 (telephone: 313-769-7100, ext 755).

Drug Delivery Systems 87

Sophia-Antipolis, France, September 14–16, 1987

For further information, contact: Mlle. Francine Canal, c/o Prof. Yves Lazorthes, Service de Neurochirurgie—CHU Rangueil, Chemin du Vallon, 31054 Toulouse Cedex, France (telephone: 33-61-53-11-33, ext 3301-3351).

The European Organization for Research and Treatment of Cancer Symposium on Recent Advances in Cancer Management

Hong Kong, September 20–23, 1987

For further information, contact: I. C. T. S., Vere and Associates, Inc, 17/F, Jubilee Commercial Bldg, 42–46 Gloucester Rd, Hong Kong (telephone: 5-279200).

2nd Certifying Examination of the American Board of Laser Surgery

San Francisco, California, October 10, 1987

For further information, contact: Mary Clayton, RN, President of the Board, 3201 South 16th St, Suite 2006, Milwaukee, WI 53215.

Second International Congress on Neo-Adjuvant Chemotherapy

Paris, France, February 4–6, 1988

For further information, contact: S.O.M.P.S, Hôpital de la Salpétrière, 47 boulevard de l'Hôpital, 75651 Paris Cédex 13, France.

Third International Conference on Adjuvant Therapy of Breast Cancer

St. Gallen, Switzerland, March 3–5, 1988

For further information, contact: Secretariat Prof. H. J. Senn, MD, Department of Medicine C, Oncology Center, Kantonsspital, CH-9007 St. Gallen, Switzerland (telephone: 719-219-KSSG CH).

Bone Marrow Transplantation: Current Controversies

Tamarron, Colorado, March 6–12, 1988

For further information, contact: UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, CA 90024 (telephone: 213-206-6292).

Third International Conference on Drug Absorption

Edinburgh, Scotland, September 28–30, 1988

For further information, contact: Dr. Ian Sword, Inveresk Research International Ltd, Musselburgh EH21 7UB, Scotland (telephone: 031-665-6881).

NCI Monographs: International Symposium on Labeled and Unlabeled Antibody in Cancer Diagnosis and Therapy

The International Cancer Information Center of the National Cancer Institute announces publication of the third issue of the new *NCI Monographs:* International Symposium on Labeled and Unlabeled Antibody in Cancer Diagnosis and Therapy (Number 3, 1987).

This volume of *NCI Monographs* contains the proceedings of a symposium held at The Johns Hopkins Hospital (Baltimore, MD), which focused on the use of antibodies in cancer diagnosis and treatment. The meeting represents the beginning of dialogue among investigators who have done research in the disciplines of physics, radiobiology, immunology, and pharmacology and those who have clinical expertise in the fields of nuclear medicine and radiation oncology. The work reported at this meeting involves use of dosimetry, low-dose-rate radiation, and antibody fragments, as well as pilot studies of isotope linkage, chemical cytotoxic antibodies, and diagnostic and therapeutic programs. These presentations demonstrate the promise of antibody therapy, which can deliver a broad range of cytotoxic agents, often without the acute side effects of other types of therapy.

Copies of this volume of *NCI Monographs* (Number 3, 1987) are available from the U.S. Government Printing Office ($9.50 per copy domestic, $11.90 foreign). To order, request Stock No. 017-042-00194-3. Make checks payable to the Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

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NIH Publication No. 87-22

April 1987